DEFINING MOLECULAR ADJUVANT EFFECTS ON HUMAN B CELL SUBSETS

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Jourdan Posner

Dissertation Committee:
Sandra P. Chang, Chairperson
George Hui
Saguna Verma
William Gosnell
Alan Katz

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ABSTRACT

Recent advances in vaccine development include the incorporation of novel adjuvants to increase vaccine immunogenicity and efficacy. Pattern recognition receptor (PRR) ligands are of particular interest as vaccine adjuvants. During early childhood, the B cell compartment contains a high frequency of immature, transitional B cells. This prominent transitional B cell population may encounter PRR ligands during immunization; however, the response of human transitional B cells to these stimuli remains largely unknown. The goal of this dissertation work was to evaluate the capacity of PRR ligands to drive transitional B cells to mature into follicular or marginal zone B cells by assessing transitional B cell maturation and evaluating the transcriptome of mature B cell subsets. This work determined that PRR ligands can drive the phenotypic maturation of human transitional B cells as measured by CD23 expression and Rhodamine 123 retention. *In vitro* transitional B cell maturation resulted in the generation of both Fo-like and MZ-like B cells as determined by surface phenotype. Additionally, select PRR ligands induced gene expression changes in transitional B cells similar to mature B cells. We analyzed human tonsil follicular and marginal zone-like B cell transcriptomes to identify several genes uniquely associated with either cell fate decision and assessed the expression of some of these genes in our *in vitro* transitional B cell maturation model. To the best of our knowledge, this is the first study to examine the differential effect of various PRR ligands on the maturation of human transitional B cells into mature, naïve B cells and to examine the follicular versus marginal zone B cell fate decision. The rational design of vaccine adjuvants that take into consideration the effect of PRR ligands on B cell maturation and differentiation may lead to new strategies to improve the immunogenicity and efficacy of childhood vaccines.

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ABBREVIATIONS

AID	Activation-induced deaminase
BCR	B cell receptor
CLR	C-type lectin receptor
CpG	CpG ODN 2006
CSR	Class switch recombination
FACS	Fluorescence-activated cell sorting
Fo	Follicular
GC	Germinal center
GLA	Glucopyranosyl lipid A
iE-DAP	y-D-Glu-mDAP
Ig	Immunoglobulin
IL-4	Interleukin-4
MHC class II	Major histocompatibility complex class II
MZ	Marginal zone
NLR	Nod-like receptor
PCR	Polymerase chain reaction
Poly (I:C)	Polyinosine-polycytidylic acid
PRR	Pattern recognition receptor
R123	Rhodamine 123
R848	Imidazoquinoline
RT-qPCR	Reverse transcriptase-quantitative polymerase chain reaction

SHM	Somatic hypermutation
T1	Transitional stage 1
T2	Transitional stage 2
T3	Transitional stage 3
TDB	Trehalose-6,6-dibehenate
TLR	Toll-like receptor

CHAPTER 1: BACKGROUND

Introduction

While vaccines are considered to be one of the most effective methods to control communicable diseases, there are no approved vaccines for many infectious diseases including malaria, HIV, dengue and West Nile virus. Candidate vaccines for malaria and dengue have provided promising results in Phase III clinical trials. The most advanced subunit malaria vaccine candidate with moderate effectiveness, RTS,S/AS01, is being assessed in several large-scale pilot implementations in sub-Saharan Africa (1). A recently published study analyzed the immunogenicity of the vaccine to assess its rate of waning (2). Differences in vaccine efficacy were age- and exposure-dependent, with anti-circumsporozoite antibodies waning rapidly in the first 6 months followed by slower waning over the next 4 years. Therefore, improved efficacy and long-term memory induction are still needed to make it a successful public health tool. One of the potential approaches to improve subunit vaccine efficacy and durability is through the use of adjuvants. While RTS,S/AS01 contains a liposome-based adjuvant system (AS01), other adjuvant systems may yield more efficacious and long lasting responses.

There are a variety of possible adjuvants in development, however, much of the rationale for choosing an adjuvant is empirical, rather than mechanistic (3). The metrics for assessing a “good” adjuvant are safety, tolerability, ability to increase vaccine potency, reduce the required antigen dose, and induce long lasting immune response (4). To date, there are a handful of adjuvant formulations licensed for use in human vaccines, of which only four are licensed for use in the US, including aluminum salts. The lack of safe and effective adjuvants is widely acknowledged as a major obstacle in modern vaccine development.

Background

Pattern recognition receptors in innate and adaptive immunity

First-generation vaccines, including inactivated/attenuated viruses or bacteria, contain inherent adjuvants that aid in immune responses to a particular pathogen. These adjuvants include pathogen components in the form of viral RNA, DNA, surface glycoproteins, lipopolysaccharides, etc. Next-generation vaccines build on the knowledge that adjuvants in vaccine formulations can differentially trigger the innate immune system and downstream signaling leading to a controlled adaptive immune response. Therefore, synthetic molecules are being designed to resemble pathogen-associate molecular patterns (PAMPs) that interact with pattern recognition receptors (PRRs), one of the main mechanisms of innate immune activation. PAMPs in clinical and experimental use as candidate vaccine adjuvants are small, immunostimulatory molecules that target cell membrane or cytosolic PRRs (5). PRRs include Toll-like receptors (TLRs), Nod-like receptors (NLRs), C-type lectin receptors (CLRs), and RIG-I-like receptors (RLRs), with expression varying on different cell types (6). TLRs are the most well-studied and well-characterized PRRs. There are 10 TLRs now described in humans, with TLR1, 2, 4-6 found on the cell membrane and TLR 3, 7-9 found on endosomal membranes. TLRs recognize a variety of bacterial and viral components: lipopeptides (TLR1/2/6), double-stranded RNA (TLR3), lipopolysaccharides (TLR4), flagellin (TLR5), single-stranded RNA (TLR7/8), and unmethylated CpG motifs (TLR9). NLRs are a family of 22 cytoplasmic receptors (7), of which NOD1, NOD2, and NLRP3 are the most well-studied. NOD1 and NOD2 recognize diaminopimelic acid (DAP)-containing muropeptides and muramyl dipeptide (MDP) groups, respectively, found

commonly on the cell wall of gram-negative bacteria (8). NLRP3 responds to a wide variety of compounds including uric acid crystals and chitosan and participates in the NLRP3 inflammasome complex (8). CLRs are expressed on the cell surface and recognize carbohydrate chains predominantly found on mycobacterial and fungal organsims (9, 10). Downstream signaling of PRR activation include pro-inflammatory cytokine secretion and chemokine expression, driving the generation of antigen depots, enhancement of antigen presentation, and upregulation of activation markers on antigen presenting cells (3).

The PRR ligands of interest to our lab and their receptors include: Polyinosine-polycytidylic acid (Poly (I:C), TLR3), Glucopyranosyl lipid adjuvant (GLA, TLR4), Imidazoquinoline (R848, TLR7/8), γ-D-Glu-mDAP (iE-DAP, Nod1), CpG ODN 2006 (CpG, TLR9), and trehalose-6,6-dibehenate (TDB, C-type lectin receptor Mincle) (Table 1). These PRRs are expressed on different immune cells in differing frequencies. The ability of PRR ligands to trigger a variety of cell types involved in an effective immune response makes them good vaccine adjuvant candidates.

Ligand	Ligand description	Pattern Recognition Receptor	PRR class	Location	Signaling Pathway
Polyinosine-polycytidylic acid (Poly (I:C))	Double-stranded RNA analog	TLR3	Toll-like receptor	Endosomal membrane	TRIF
Glucopyranosyl lipid A (GLA)	Non-toxic synthetic lipid A	TLR4	Toll-like receptor	Cell surface membrane	TRIF/MyD88
Imidazoquinoline (R848)	Imidazoquinoline compound	TLR7/8	Toll-like receptor	Endosomal membrane	MyD88
CpG ODN2006 (CpG)	Oligondeoxyucleotides	TLR9	Toll-like receptor	Endosomal membrane	MyD88
y-D-Glu-mDAP (iE-DAP)	Peptidoglycan subunit	NOD1	Nod-like receptor	Cytosolic	RIP2
Trehalose-6,6-dibehenate (TDB)	Synthetic trehalose dimycolate/cord factor	Mincle	C-type lectin receptor	Cell surface membrane	FcRg/CARD9

Table 1. Pattern recognition receptors of interest and their respective ligands. Additional information including receptor class, location and signaling pathway are noted.

Human B cell populations

Germinal center formation is key to the production of high affinity antibodies and B cell memory (11), two critical elements of immunity to many viral, bacterial, and protozoan pathogens, including malaria. Transitional, marginal zone (MZ), and follicular (Fo) B cells contribute to the development, structure, and function of germinal centers (12). Transitional B cells are recent emigrants of the bone marrow. These cells have successfully passed central tolerance in the bone marrow and express a properly formed B cell receptor, however, they are still considered immature. Three stages of transitional B cells have been identified in the human based on cell surface markers: T1, T2, and T3, of which T1 B cells are the most immature. Transitional B cells are highly susceptible to BCR-induced apoptosis and exhibit poor ex vivo survival due to their functional immaturity (13). Upon maturation in the periphery, transitional B cells differentiate into MZ or Fo B cells localized to secondary lymphoid organs. The exact mechanism and timing of B cell fate decisions is not completely understood, however, the role of BCR signal strength and Notch signaling have been suggested (14). It is commonly proposed that a strong BCR signal supports Fo B cells while a weak BCR signal favors MZ B cells. Additionally, the Notch2 signaling pathway participates in MZ B cell commitment. The spleen is the only secondary lymphoid organ with a true marginal zone, although marginal zone-like regions have been identified in Peyer's patches and tonsils. In the spleen, MZ B cells border the perifollicular zone and due to their close proximity to peripheral circulation act as a first line of defense to blood borne pathogens. MZ B cells are constantly in a pre-activated state allowing their rapid differentiation into IgM-secreting plasma cells in response to BCR crosslinking. MZ B cells can also bind

and shuttle antigen into the B cell follicle (15). The majority of transitional B cells, however, mature into Fo B cells, responsible for T-dependent antigen responses and differentiation into memory B cells or plasma cells. In addition, MZ and Fo B cells have been implicated in the differentiation and maintenance of T follicular helper (Tfh) cells (16), the limiting factor in the maturation of high affinity B cells.

MZ B cell controversy

Recent literature has demonstrated that human MZ B cells have variable surface marker expression and tissue distribution. It is therefore recognized that the human MZ B cell population is heterogeneous but shares three common characteristics: 1) the ability to recirculate, 2) CD27 expression, and 3) the presence of mutated VDJ genes (17-19). Since these three characteristics are hallmarks of memory B cells, there has been a debate over whether MZ B cells are a subset of memory B cells or represent an independent B cell lineage. One hypothesis states that MZ B cells are a distinct subpopulation that arise in a germinal center-independent manner (20-22). The alternative hypothesis states that MZ B cells are post-germinal center memory cells that have retained IgM expression, rather than switching to another immunoglobulin isotype (22). Most literature identifies human MZ B cells as IgM^+ IgD^+ $CD27^+$, referring to these cells as IgM memory B cells. Evidence for both hypotheses will be discussed.

Several immunodeficiencies including hyper-IgM syndrome, X-linked lymphoproliferative syndrome, and common variable immune deficiency result in absent or aborted GC formation (23-25). In these patients, although GCs are absent and/or aborted, IgM^+ IgD^+

$CD27^+$ MZ B cells are still found. This supports the hypothesis that MZ B cells can form in a GC-independent manner. Additionally, antigen-specific IgM memory B cells can form in response to T-dependent antigens in mouse models where GC formation is prevented, including Bcl-6-deficient bone marrow (26) and $CD28^{-/-}$ mice (27). Although the mutation frequency of these B cells is lower in these mice as compared to wild type, their presence indicates that GCs are not necessary for MZ B cell development.

A number of other studies provide evidence in support of the alternative hypothesis that MZ B cells are post-GC memory cells. Somatic hypermutation (SHM) occurs prior to isotype switching in GCs (28), therefore, it is possible that abortive GCs can give rise to IgM^+ memory B cells. Additionally, most IgM^+ $CD27^+$ B cells have undergone less proliferation than switched memory B cells (29), which is indicative of an early exit from the GC, as seen in abortive GC formation.

To refute the theory that abortive GCs may give rise to mutated MZ B cells, studies have shown that MZ B cells undergo SHM during the generation of the pre-immune repertoire, supporting the hypothesis that MZ B cells are a distinct subpopulation that develops in a GC-independent manner (30). The expression of activation-induced cytidine deaminase (AID), an enzyme required for the induction of SHM, has been observed in immature murine B cells with somatically mutated Ig V genes (31). In immature B cells, SHM is necessary for the removal and tolerization of autoreactive B cells prior to GC entry (32). These studies suggest that B cells can undergo SHM

outside of GCs. Since B cells in the marginal zones contain little to no AID, these cells must have undergone SHM prior to entering the MZ (33).

IgM^+ $CD27^+$ MZ B cells are critical for responses to T-cell independent (TI) antigens. Asplenic children and adults have low frequencies of IgM^+ $CD27^+$ MZ B cells, which correlates with increased frequency of encapsulated bacterial infections. However, switched memory B cells are also found at a lower frequency in asplenic children and adults. Therefore, the response to TI antigens may be contributed from both IgM^+ $CD27^+$ MZ and switched memory B cells. If IgM^+ $CD27^+$ MZ B cells are a distinct subpopulation critical for TI antigens, this population should be enriched for clones specific for bacterial polysaccharide TI antigens. Tsuiji et al. (34) showed that less than 4% of IgM^+ $CD27^+$ MZ B cells are polyreactive to TI bacterial antigens as compared to more than 10% of mature naïve B cells. Additionally, IgM^+ $CD27^+$ MZ B cells have been shown to efficiently respond to T-cell dependent antigens. Together, these data suggest that IgM^+ $CD27^+$ MZ B cells are not the only subpopulation critical for TI responses.

Aranburu et al. (35) studied paired peripheral blood and splenic samples from children of different ages to understand how age- and tissue-specific events shape the memory B cell compartment including IgM^+ $CD27^+$ MZ B cells. They proposed that, in addition to switched memory B cells, three types of IgM memory B cells exist: innate IgM memory B cells, remodeled IgM memory B cells, and GC-derived IgM memory B cells. Innate IgM memory B cell development is GC-independent, spleen-dependent and found primarily in infants. They have a low mutation frequency and their immunoglobulin

repertoire is quasi-germline. If the individual has a spleen, innate IgM memory B cells have the potential to undergo somatic hypermutation in a GC-dependent manner to become remodeled IgM memory B cells. Remodeled IgM memory B cells are found in older children. The third type of IgM memory B cell is GC-derived IgM memory B cells. Found in both infants and older children, GC-derived IgM memory B cells do not require the spleen but do require GCs and IL21 for SHM to occur. In comparison to innate IgM memory B cells, GC-derived IgM memory B cells have restricted Ig repertoires. The finding of three types of IgM memory B cells indicates that the MZ may change with age consisting of innate IgM memory B cells at infancy and GC-experienced IgM memory B cells during adulthood.

PRR expression on human B cell populations

PRR ligands can trigger the activation and differentiation of a variety of cells. Of particular interest to an effective immune response is the activation and differentiation of B cell subsets important to functional germinal centers. PRRs are expressed on human B cell subsets, however, information on PRR expression is limited as most analyses are done with the B cell population as a whole (9, 36, 37) which is not a homogenous population. Often B cells from peripheral blood are analyzed because of accessibility. Unfortunately, the major B cell populations involved in antigen processing, presentation and proper germinal center formation are localized to the draining lymphoid tissues. Additionally, PRR expression is known to alter in response to various stimuli, such as BCR and/or CD40 ligation. For example, resting naïve B cells have relatively low TLR expression, however, in response to BCR ligation several TLRs are upregulated (38).

The variability between B cell subsets and differences in activation states complicates much of the PRR expression data. Table 2 is a summary of select PRRs and their expression on various human B cell subsets.

		Transitional	Follicular/ Naïve [20]	Marginal Zone	Germinal Center [21]	Memory (from tonsil) [20]	Total Circulating CD19+ [22]
Toll-like Receptors	TLR 1		+		++	+	++
	TLR 2		+/-		-	+	+
	TLR 3		-		-	-	-
	TLR 4		+/-		-	-	+/-
	TLR 5		+/-		-	+	-
	TLR 6		++		++	+	++
	TLR 7		++		++	+	+
	TLR 8		+/-		+	-	-
	TLR 9	+ [23, 24]	+		++	+	+
	TLR 10		+		++	+	+
Nod-like Receptors	NOD1		+ (tonsil B cells) [17]	+ (tonsil B cells) [17]			++
	NOD2		- (tonsil B cells) [17]	- (tonsil B cells) [17]			+
C-type lectin Receptor	Mincle						Naïve: ++ Memory: + [9]

Table 2. Pattern recognition receptor expression on human B cell subsets.

Positive expression is measured by mRNA and/or protein detection.

PRR role in B cell maturation and activation

Several studies have implicated a role for PRR signaling in B cell maturation. TLR9 ligation of human transitional B cells has been shown to drive terminal differentiation to a marginal zone phenotype and secretion of IgM antibodies (39, 40). Others have demonstrated the role of TLR signaling in germinal center regulation (41). Various studies have shown that mouse and human B cells increase co-stimulatory molecule expression, secrete IgM antibodies, and proliferate in response to different PRR ligands (39, 42-45).

Incorporation of adjuvant alters the immune response

Adjuvant-specific immune responses are being extensively studied (6, 46-48). Knudsen et al. performed a comparative analysis of five clinical-grade adjuvants (Alum, MF59, GLA-SE, IC31, CAF01) with three model antigens from *M. tuberculosis*, influenza, and Chlamydia (46). This analysis allowed the direct comparison of each adjuvant's immune profile in human PBMCs and the immune profile changes depending on the antigen. Emulsion-based adjuvants like Alum and MF59 favor Th2 responses, while adjuvants that contain PRR agonists (CAF01 – Mincle agonist, IC31 – TLR9 agonist) favor Th1 responses. GLA-SE is an interesting adjuvant because of the balanced Th1/Th2 profile it elicits in mice. GLA (glycopyranosyl lipid adjuvant) is a TLR4 agonist that alone induces a polarized Th1 response in mice. However, when GLA is formulated with SE (a stable emulsion of oil-in-water), a potent Th1 response is balanced with enhanced antibody titers (49). With a better understanding of adjuvant mechanisms of action and

their resulting immune signatures, we can design vaccines using a mechanistic rationale.

Malaria vaccines and their adjuvants – efficacy and responses

Over the past 30 years, more than 25 different malarial antigens in formulation with more than 15 different adjuvants have been proposed.

Year	Model	Antigenic Component	Adjuvant(s)
1989	Human	(NANP)3-TT	IFN alpha/gamma
1990	Human	Irradiated sporozoites	None
1990	Monkey	p41/190N	CFA/IFA
1990	Monkey	S7+S12+S17+SPf66	CFA/IFA
1992	Human	SPf66	Alum
1992	Monkey	E coli derived fusion proteins (MSAI, SERP, HRPII)	Alum
1992	Monkey	E coli derived fusion proteins (MSAI, SERP, HRPII)	Oil-based emulsion
1992	Monkey	Recombinant DNA-encoded fragment (C7Ag) of Pf p75	Syntex adjuvant formulation/alum
1993	Monkey	SERA 1	CFA/IFA, MF59.2, MF75.2
1995	Monkey	SPf66	Alum
1995	Monkey	MSP1(42) and MSP1(19)	Freund's
1995	Human	SPf66	Alum
1996	Monkey	NYVAC-Pf7, attenuated vaccinia virus expressing CSP, SSP2, LSA1, MSP1, SERA, AMA1, Pfs25	None
1996	Human	SPf66	Alum
1997	Human	RTS,S	Alum+MPLA/oil in water emulsion/emulsion+MPLA+QS21

Table 3. Summary of malaria vaccines in development by year. This list of malaria vaccine candidates is the result of an extensive PubMed literature search using the key terms “malaria”, “vaccine”, and “adjuvant” from 1989 to 2018. The animal model, vaccine antigen, and adjuvant(s) included are listed.

Year	Model	Antigenic Component	Adjuvant(s)
1997	Monkey	Liver stage antigen 3	Freund's
1997	Mouse	MSA-2	Montanide ISA 720/SAF-1/liposomes with lipid A
1998	Mouse	AMA1	Montanide ISA 720/Freund's
1999	Human	MSP1 fragment+MSP2+RESA fragment	Montanide ISA 720
1999	Monkey	Pf (T1B)4 multiple antigen peptides	Freund's/alum/QS21
2002	Mouse	Recombinant BCG expressing CSP	None
2002	Mouse	MSP1(19)+MSP4/5	Freund's
2003	Human	RTS,S	AS02
2003	Mouse	SPf66	alum/PLGA microparticles
2003	Monkey	MSP1(42) and MSP1(19)	Freund's
2003	Human	MSP1+MSP2+RESA	Montanide ISA 720
2004	Mouse	MSP1(19)+MSP4/5	CpG ODN1826/Montanide ISA 51
2004	Human	PfCSP DNA prime + RTS,S/AS02A boost	AS02A
2004	Mouse	Pan HLA DR-binding epitope carrier for MSP1(19)	
2004	Monkey	RTS,S+TRAP	AS02A
2004	Mouse	Recombinant Pv Duffy binding protein	Montanide ISA 720/AS02A/Alum
2004	Monkey	MSP1(42) and MSP1(19)	AS01B/AS02A/AS05/AS08
2004	Mouse	PcAMA and/or MSP1(42)	Quil A
2004	Human	Modified hepatitis B virus core protein ICC-1132 (Malarivax)	Alum
2005	Mouse	EB200 DNA construct	PfHSP70C
2005	Monkey	PfGRP and PfMSP3	Freund's/Montanide ISA 720
2005	Human	PfAMA1	Montanide ISA 720
2005	Human	ICC-1132	Montanide ISA 720
2005	Human	RTS,S	AS02A
2005	Monkey	ICC-1132	Alhydrogel/Montanide ISA 720
2006	Human	FMP1	AS02A
2006	Mouse	PfMSP1(42)	Montanide ISA 720/alum/MF59
2007	Human	FMP1	AS02A
2006	Mouse	AMA1-C1	Alhydrogel+CpG 7909
2007	Human	PfGLURP	Alum/Montanide ISA 720
2007	Mouse	Recombinant F2(PfEBA-175)	Montanide ISA 720/AS02A/Alum
2007	Mouse	Recombinant LSA1	AS01B/AS02A

Table 3 (continued). Summary of malaria vaccines in development by year.

Year	Model	Antigenic Component	Adjuvant(s)
2006	Monkey	Recombinant Pfs25H conjugated to OMP complex (N. meningitidis serogroup B)	Alum
2007	Monkey	Replication-defective human Ad35.CS prime + RTS,S/AS01B boost	AS01B
2007	Human	FMP2.1	AS02A
2008	Human	PfLSA1 alone or concurrently with RTS,S	AS01B
2008	Human	AMA1/MSP1 chimeric protein	Montanide ISA 720
2008	Mouse	AMA1+Pfs25 conjugated to P. aeruginosa ExoProtein A	Alhydrogel +/- CpG 7909
2008	Human	Pfs25+Pvs25	Montanide ISA 51 - water in oil emulsion
2008	Human	AMA1-C1	Alhydrogel+CpG 7909
2008	Mouse	FALVAC-1A	Alum/QS21/Montanide ISA 720/copolymer CRL-1005
2008	Mouse	MSP1(19)	S. enterica serovar Typhimurium flagellin-TLR5 agonist (mixed or linked)
2009	Mouse	PfCS peptides subQ	topical imiquimod
2008	Human	RTS,S	AS01E
2008	Human	PfAMA1	Alhydrogel/Montanide ISA 720/AS02
2008	Mouse	PbCSP	modified E. coli heat-labile toxin
2009	Mouse	PfMSP4/5	CFA versus AFCo1
2009	Human	RTS,S	AS02
2009	Human	AMA1	AS01B/AS02A
2009	Human	AMA1-C1	Alhydrogel+CpG 7909
2009	Monkey	PvRAP2	Freund's/Alum
2009	Human	AMA1-C1	Alhydrogel
2010	Human	LSA-NRC	AS01/AS02
2009	Monkey	PvMSP10	Freund's/Montanide ISA 720/Alum
2009	Human	RTS,S	AS02D/AS01E
2009	Human	AMA1-C1	Alhydrogel+CpG 7909
2010	Mouse	PfMSP-Fu(24) chimeric protein from MSP1(19) and MSP3(11)	CFA/IFA/Montanide ISA 720/Alum
2010	Mouse	Recombinant Pvs25	non-toxic cholera toxin B subunit delivery system
2010	Mouse	Pf332-DBL	Montanide ISA 720/alum/levamisole
2010	Human	EBA-175	Alum
2010	Monkey	PfCSP or antiDEC-CSP	Poly (I:C)
2011	Mouse	GLURP-MSP3	GLA

Table 3 (continued). Summary of malaria vaccines in development by year.

Year	Model	Antigenic Component	Adjuvant(s)
2011	Monkey	PkAMA1	CoVaccine HT
2011	Monkey	VMP001 chimeric recombinant PvCSP	GLA-SE
2011	Human	EcMSP2-3D7 and EcMSP2-FC27	Montanide ISA 720
2012	Human	VAR2CSA DBL4e-ID4	Montanide ISA 720/Alhydrogel/CAF01
2011	Mouse	Virosomes with MSP3 and GLURP	Alum/Montanide ISA 720
2012	Mouse	Recombinant BCG expressing SE22 from the N terminal domain of SERA	None
2012	Human	BSAM2 (FVO and 3D7 alleles of rAMA1 and rMSP1(42)	Alhydrogel+CpG 7909
2013	Monkey	SE36/AHG	K3 ODN/D3 ODN/synthetic hemozoin
2013	Human	PfFVO MSP1(42)	AS01
2013	Mouse	LbL polypeptide films on solid CaCO3 cores containing a tri-epitope CS peptide	Pam3Cys
2013	Mouse	Pfs25H-EPA conjugated nanoparticle	Alhydrogel
2014	Human	RTS,S+TRAP	AS02
2014	Mouse	Recombinant CSP from Pseudomonas fluorescens	
2015	Human	JAIVAC-1 (rPfMSP1(19) + rEBA175)	Montanide ISA 720
2015	Mouse	Library of CS epitopes on woodchuck hepatitis virus core antigen VLP	IFA/Alum
2016	Human	FMP2.1 (3D7 AMA1)	AS01
2016	Mouse	E coli OMV expressing AnAPN1 and Pfs48/45	Intranasal: Cholera Toxin/Parenteral: MF59C.1
2017	Mouse	PfUIS3 in viral vectors ChAd63/MVA with PfTRAP	
2017	Mouse	FMP014 (60 identical monomber protein chains that self-assemble into nanoparticles)	Army Liposomal Formulation (ALF; liposomal adjuvant containing MPLA)
2017	Mouse	PvTRAP-VLP	Microcrystalline tyrosine
2017	Mouse	FMP013 (nearly full length CSP from 3D7)	ALF alone/ALF+Alum/ALF+QS21/Montanide ISA 720
2017	Mouse	Full length rPfCSP	CAF09
2017	Human	PfAMA1-DiCo	GLA-SE/Alhydrogel
2017	Mouse	Self-assembling protein nanoparticles with PfCSP	TLR5 agonist flagellin
2018	Mouse	PfMSP1(42)	Poly (I:C)/CFA/IFA

Table 3 (continued). Summary of malaria vaccines in development by year.

The most employed adjuvants in developing malaria vaccines are aluminum salts, Montanide ISA 720, and Adjuvant Systems AS01/AS02. One reason for biased adjuvant incorporation is that these adjuvants are also delivery systems for the vaccine antigen. Another reason may be their availability and well-defined tolerability. Aluminum salt formulations and Montanide ISA 720 are readily available for purchase, and although GSK's Adjuvant Systems are proprietary, they collaborate with developers of multiple malaria vaccines candidates. Additionally, several malaria vaccine formulations incorporate a TLR ligand (50-54).

The RTS,S vaccine, the most advanced malaria vaccine candidate to date, has undergone several revisions to the adjuvant formulation. Early formulations of RTS,S compared three adjuvants: Alum/MPLA (TLR4 agonist), oil-in-water emulsion, oil-in-water emulsion plus MPLA/QS-21 (saponin derivative) (55). Circumsporozoite antibody titers were most elevated following three doses of RTS,S with the oil-in-water emulsion plus MPLA/QS-21. It is now appreciated that emulsion-based adjuvants induce Th2 responses associated with elevated antibody titers. For the next decade of evaluation, the RTS,S vaccine adjuvant formulation contained similar components, an a-tocopherol/squalene oil-in-water emulsion with MPL/QS-21 (also known as AS02). In 2008, AS01, a more immunogenic adjuvant containing liposomes instead of an oil-in-water emulsion, was introduced with RTS,S (56). The vaccine efficacy of RTS,S/AS01 (53%) was promising in comparison to the previous RTS,S/AS02 formulation (30%). RTS,S/AS01 is the current vaccine formulation employed in large-scale pilot studies (2). The phase III trial demonstrated the RTS,S/AS01 vaccine efficacy to be less than 30%.

It is clear that this vaccine alone will need additional interventions to be effective. Future work will require revisions to the vaccine adjuvant formulation.

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CHAPTER 2: DISSERTATION SCOPE

Background

PRR ligands are small molecules that target either cell membrane or cytosolic receptors of the innate immune response, triggering various signaling cascades and, ultimately, activating the adaptive immune response (1). The ability of these PRR ligands to stimulate a variety of cell types required for an effective immune response makes them good vaccine adjuvant candidates (2). The PRR ligands of interest to our lab as potential vaccine adjuvants and their receptors include: Polyinosine-polycytidylic acid (Poly (I:C), TLR3), Glucopyranosyl lipid A (GLA, TLR4), Imidazoquinoline (R848, TLR7/8), γ-D-Glu-mDAP (iE-DAP, Nod1), CpG ODN 2006 (CpG, TLR9), and trehalose-6,6-dibehenate (TDB, C-type lectin Mincle).

Germinal center formation is key to the production of high affinity antibodies and B cell memory (3), two critical elements of immunity to many viral, bacterial, and protozoan pathogens, including malaria. Our lab is interested in the effects of PRR ligands on B cell subsets important to the formation of functional germinal centers. These B cell subsets include transitional, marginal zone (MZ), and follicular (Fo) B cells which contribute to the development, structure, and function of germinal centers. While Notch signaling plays a role in certain B cell fate decisions (4), the broader effects of PRR ligands on gene expression during early stages of B cell maturation are not fully understood. In addition, MZ and Fo B cells have been implicated in the differentiation and maintenance of T follicular helper (Tfh) cells and the interaction between B cells and Tfh cells constitutes a limiting step in the selection of high affinity B cells.

Various infections, including malaria, disrupt B cell lymphopoiesis. During malaria infection, transitional and MZ B cells are among the populations disturbed (5-7), however, how their functional properties may be affected has not been fully elucidated.

The overall goals of our project are to evaluate the capacity of PRR ligands to drive the maturation, differentiation, and activation of B cell subsets. We hypothesize that select PRR ligands will effectively drive the maturation and activation of B cell subsets. These PRR ligands will be idea adjuvant candidates for vaccines requiring a robust antibody response. Additionally, information gained about the effects of PRR ligands on human B cells will aid in future rational design of vaccine adjuvants.

Specific Aim 1. Identify the direct effects of PRR ligands on human B cell development.

PRR ligands can bind to receptors on human B cells at a variety of developmental stages, including immature transitional B cells, mature follicular B cells and marginal zone B cells. In Specific Aim 1A, we will determine which PRR ligands are capable of driving the maturation of transitional B cells into Fo or MZ B cells and how these PRR ligand effects may be altered by BCR ligation. In Specific Aim 1B we will study the ability of PRR ligands with and without BCR ligation to activate mature B cells. PRR ligands that drive follicular B cell maturation and activation will be ideal adjuvant candidates for vaccines requiring long-lived memory and antibody responses, as the follicular B cell subset represents the precursors of germinal center B cells.

Hypothesis

We hypothesize that select PRR ligands will drive transitional B cells to mature into Fo B cells, while others will drive MZ B cell maturation.

Approach

Specific Aim 1A. Identify PRR ligands that drive the differentiation of transitional B cells into marginal zone-like (MZ-like) or follicular (Fo) B cells

The goal of this aim is to use flow cytometry to isolate three B cell subsets (transitional, marginal zone-like and follicular) from human primary mononuclear cells and analyze the ability of PRR ligands to drive their differentiation. Transitional B cell subsets (T1, T2, T3) will be characterized by surface antigen expression in human cord blood mononuclear cells (Hemacare, Van Nuys, CA) and human peripheral blood mononuclear cells from adult volunteers. Transitional B cells will be isolated from human cord blood mononuclear cells by flow cytometry as $CD19^{+}$ $CD24^{+/hi}$ $CD38^{+/hi}$ Rhodamine 123^{+} cells (8). Sorted transitional B cells will be cultured in the presence of IL-4, a known survival factor for human transitional B cells, with or without PRR ligands and anti-IgM. Following culture for 2-4 days, cells will be analyzed by flow cytometry to determine the percentage of cells with a MZ-like ($CD19^{+}$ $CD21^{+/-}$ $CD23^{-}$) or Fo ($CD19^{+}$ $CD21^{+/-}$ $CD23^{+}$) B cell phenotype. Rhodamine 123 retention will be used to identify B cells that remain immature.

Specific Aim 1B. Identify PRR ligands that activate tonsillar MZ-like or Fo B cells.

The goal of this aim is to use flow cytometry to isolate marginal zone-like and follicular B cells from human primary mononuclear cells and analyze the ability of PRR ligands and

BCR ligation to drive their activation. Human tonsils (Kapiolani Medical Center, Honolulu, HI) will be obtained and subsequently dissociated for FACS sorting. Cells will be stained for MZ-like and Fo B cell surface markers as described above and sorted into two separate populations. Sorted MZ-like and Fo B cells will be cultured overnight with individual PRR ligands with and without anti-IgM. Following overnight culture, cells will be recovered and costimulatory/activation molecules (CD86 and MHCII) and immunoglobulin expression will be analyzed.

Specific Aim 2. Evaluate the transcriptomic profiles of human B cell subsets

Several signaling pathways and transcription factors have been associated with B cell maturation from immature transitional B cells to mature naïve follicular and marginal zone B cells. However, to date, the transcriptomic profiles of human transitional, follicular and marginal zone B cells have not been evaluated concurrently. Specific Aim 2A will analyze the transcriptome of these B cell subsets. Notch signaling and E proteins have been reported to play a key role in the cell fate determination of murine mature B cells, in Specific Aim 2B we will determine whether these genes also play a key role in human mature B cells. In Specific Aim 2C, we will evaluate the ability of PRR ligands to initiate Notch signaling as well as signaling through other pathways that potentially affect B cell maturation of immature transitional B cells into mature follicular or marginal zone B cells.

Hypothesis

We hypothesize that genes involved in Notch signaling, murine B cell maturation, and select pathways identified following transcriptome analysis will characterize mature human Fo and MZ B cells. These genes will be differentially expressed following the stimulation of immature transitional B cells with select PRR ligands.

Approach

Specific Aim 2A. Transcriptome analysis of transitional, follicular, and marginal zone B cells

The goal of this aim is to use flow cytometry and AmpliSeq technology to analyze the transcriptome of human cord blood transitional B cells, human tonsillar follicular B cells and human tonsillar marginal zone B cells. Each B cell subset will be isolated by flow cytometry and RNA will be extracted for downstream library preparation and RNA sequencing. Bioinformatics analysis of differential gene expression will be assessed to identify genes associated with human B cell lineage commitment. Raw reads will be quality controlled and aligned to the human genome using STAR. Differential expression will be analyzed using the DESeq2 software package, visualized using R and pathway and network analysis will be performed using Ingenuity Pathway Analysis (IPA).

Specific Aim 2B. Gene expression analysis of tonsillar follicular and marginal zone B cells

The goal of this aim is to use real-time qPCR to compare the expression of Notch signaling components and maturation genes of human tonsillar follicular and marginal

zone B cells to their murine splenic counterparts. Following FACS isolation of follicular and marginal zone B cells according to their previously stated cell surface markers, RNA will be isolated, and cDNA will be synthesized. Real-time qPCR will be performed using primers for 15 genes encoding B cell specific Notch signaling components and 7 genes involved in B cell maturation.

B cell-specific Notch signaling genes					**B cell maturation genes**	
ADAM10	HES5	JAG1	NCSTN	NUMB	BTK	ASB2
GLI1	HEY2	LFNG	NOTCH2	RBPJL	ID2	TCF3
HES1	HEYL	NCOR	NOTCH2NL	SNW1	ID3	

Table 1. List of genes evaluated in B cell populations by real-time qPCR.

Specific Aim 2C. Gene expression analysis during transitional B cell differentiation.

The goal of this aim is to use flow cytometry and real-time qPCR to evaluate the role of Notch signaling and other lineage commitment genes identified by AmpliSeq analysis during normal B cell development and determine whether PRR ligands can skew B cell development by directly affecting a subset of these genes. Human transitional B cells will be FACS-sorted according to their previously stated cell surface markers. Following culture with PRR ligands, RNA will be isolated, and cDNA will be synthesized. Control cultures will include no PRR stimulation. To analyze the relative expression of Notch signaling transcripts, cDNA will be added to PrimePCR Array plates containing primers specific for more than 40 gene targets important to the Notch signaling pathway. Real-

time qPCR will also be used to evaluate expression of other B cell maturation-associated genes identified in the RNA Seq studies.

Human Subjects Research

For Aim 1 & 2A-C, human peripheral blood, cord blood and tonsils will be used. Human peripheral blood is obtained following informed consent. No identifying information will be collected. Human cord blood is purchased from a commercial vendor. Human tonsils are provided as exempt tissues from routine tonsillectomies by our clinical collaborator, Dr. Patrick O'Donnell at Kapiolani Medical Center. No identifying information is provided. This study has been reviewed and approved by the University of Hawaii Human Studies Program.

Relevance

Small molecular adjuvants are in clinical and experimental use (9), however, their effects on specific human B cell subsets are not fully understood. Since a vaccine's goal is to establish a robust antibody response, it is important to understand the ability of the vaccine adjuvant to drive B cell development and activation. Adjuvants may skew the resulting immune response by acting directly on B cell functions or indirectly by affecting antigen presenting cells and T helper cell development. This proposal will investigate the direct effects of select molecular adjuvants on human B cell subsets. Understanding adjuvant effects on B cell development and function is essential when selecting an ideal vaccine adjuvant.

Significance

While vaccines are considered to be one of the most effective methods to control communicable diseases, there are no approved vaccines for many infectious diseases including malaria, HIV/AIDS, dengue and West Nile virus (10). Candidate vaccines for malaria and dengue have provided promising results in Phase III clinical trials. The most advanced malaria vaccine candidate with moderate effectiveness, RTS,S/AS01, is being assessed in several large-scale pilot implementations in sub-Saharan Africa (11). A recently published study (12) analyzed the immunogenicity of this vaccine over time to assess the waning of immunity. Differences in vaccine efficacy were age- and exposure-dependent, with anti-circumsporozoite antibodies waning rapidly in the first 6 months followed by a slower decline over the next 4 years. Therefore, improved efficacy and long-term memory induction is still needed to make this vaccine a successful public health tool. One of the potential approaches to improve subunit vaccine efficacy and durability is through the use of adjuvants. While RTS,S/AS01 consists of a liposome-based adjuvant system containing monophosphoryl lipid A (MPL) and the saponin QS-21, other adjuvant systems may yield more efficacious and longer-lasting responses. There are a variety of possible adjuvants in development, however, much of the rationale for choosing an adjuvant is empirical, rather than mechanistic (13). To date, there are only a handful of adjuvant formulations licensed for use in human vaccines, of which four are licensed for use in the US, including alum (14). The lack of safe and effective adjuvants is widely acknowledged as a major obstacle in modern vaccine development.

PRR ligands and B cells

Vaccine adjuvant candidates to date include PRR ligands, oil-in-water emulsions, saponins, liposomes, and nanoparticles, alone or in combination (15). PRR ligands are small, immunostimulatory molecules that target either cell membrane or cytosolic receptors of the innate immune response, trigger various signaling cascades leading to an activated adaptive immune response (2). Various studies have shown that mouse and human B cells increase co-stimulatory molecule expression, secrete IgM antibodies, and proliferate in response to different PRR ligands (16-20). Of interest to our lab are the effects of these PRR ligands on B cell subsets important to functional germinal centers. Germinal center formation is key to the production of high affinity antibodies and B cell memory (21), two critical elements of immunity to many viral, bacterial, and protozoan pathogens, including malaria. Transitional, marginal zone (MZ), and follicular (Fo) B cells contribute to the development, structure, and function of germinal centers and extrafollicular immune responses (22). In addition, MZ and Fo B cells have been implicated in the differentiation and maintenance of T follicular helper (Tfh) cells (23), the limiting factor in the maturation of high affinity B cells. Current information on PRR expression of human B cell subsets is limited as most analysis is done with the B cell population as a whole (24-26).

B cell subsets of the human tonsil

Human palatine tonsils are secondary lymphoid organs of the nasopharynx-associated lymphoid tissue (27, 28). Secondary lymphoid organs contain B cell follicles that develop into germinal centers, sites of intense B cell proliferation, affinity maturation,

and isotype class switching in response to vaccination or infection (21). The B cell populations present in the human tonsil include: follicular B cells, marginal zone-like B cells, germinal center B cells, newly formed memory B cells and plasmablasts (29). While localized to the tonsil at the time of vaccination or infection, the resulting memory B cells and plasmablasts can recirculate systemically and produce high affinity antibodies.

Innovation

Need to define adjuvant effects on human B cell subsets

The vast majority of research examining the effects of PRR ligands on B cell subsets has been done using the mouse model. While some work has been done examining the effects of PRR ligands on B cells in humans, most of that work examines the effects of PRR ligands on the B cell population as a whole (25, 30). Yet, the B cell population is very heterogeneous. Functionally distinct subsets can reside in the same tissue while similar B cell subsets will have different functionalities in different tissues. This project will tease out the different effects PRR ligands have on human B cell subsets present in cord blood and the tonsils.

Human cord blood and tonsils as B cell sources

Human cord blood contains approximately 5-10% $CD19^+$ B cells with the majority of those having a transitional B cell phenotype. This is the most enriched source of human transitional B cells available. In comparison, approximately 5-20% of lymphocytes in adult peripheral blood are B cells, of which only about 4% are transitional B cells.

Tonsils are the most accessible human secondary lymphoid organs. B cells represent approximately 80% of tonsil lymphocytes. Five B cell subsets based on IgD and CD38 expression are present in human tonsils: naïve B cells (~30%), pre-germinal center B cells (~10-15%), germinal center B cells and plasmablasts (~40%), and memory B cells (<10%). Therefore, human cord blood and tonsils are ideal B cell sources.

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CHAPTER 3: PRR LIGANDS DRIVE HUMAN TRANSITIONAL B CELL MATURATION

1. Introduction

While vaccination is recognized as one of the most effective approaches for infectious disease prevention, there is a pressing need for safe and effective immune modulators also known as “adjuvants”, to enhance the effectiveness and durability of many current and experimental vaccines, particularly for the very young and the elderly (1, 2). The identification of pattern-recognition receptor (PRR) ligands or pathogen-associated molecular patterns (PAMPS) as potent immune modulators has led to their evaluation as vaccine adjuvants. Some vaccines contain naturally occurring PAMPS like viral nucleic acids in the Yellow Fever vaccine, while others utilize additional PRR ligands like MPL, a TLR4 ligand adsorbed to alum, in the recombinant HPV vaccine Cervarix (3, 4). Much of the previous research investigating the cellular effects of PRR ligands has focused on their effects on antigen-presenting cells (5-7), however B cells also are potential responders to PRR stimulation and are of interest as direct targets of adjuvant immunomodulation.

Mechanistic studies of PRR ligand adjuvant activity in the mouse model have provided important insights into the influence of PAMPS on the immune response, however mechanisms defined in the mouse model often do not translate directly to humans (8, 9). In addition, the spectrum of potential PRR receptors has increased in recent years and now includes Toll-like receptors (TLRs), Nod-like receptors (NLRs), and C-type lectin receptors (CLRs), among others (10). While the effects of a few PRR ligands on human B cells have been explored (11, 12), knowledge of their contribution to the

differentiation and activation of B cell subsets important to germinal center formation, a critical feature of effective antibody-mediated immunity, is limited.

Transitional B cells are important intermediates in the development of mature B cells (13). Transitional B cells are immature B cells that have recently migrated from the bone marrow to the periphery following expression of a functional B cell receptor (14). After selection and further differentiation, they give rise in secondary lymphoid organs to mature B cell populations, including follicular (Fo) B cells and marginal zone (MZ) B cells. Fo B cells play a central role in germinal center formation in response to T cell-dependent antigens, and are responsible for the production of high-affinity, class-switched antibody, and differentiate into long-lived plasma cells and memory B cells (15). MZ B cells respond to blood borne pathogens and T-independent type 2 antigens, differentiate into short-lived plasma cells, and also play a role in antigen capture and shuttling to follicular dendritic cells (16, 17).

Total B cell and B cell subset frequencies vary depending on an individual's age. In addition to the gradual decrease in total B cell count from newborn to six years of age, there is a switch in prominent B cell populations from mostly immature/transitional B cells in early childhood to mature and memory B cells in young adults (18). Since many current vaccines and vaccines in development are administered during childhood, it is important to examine the effects of vaccine components like PRR ligands on the prominent transitional B cell population in newborns and young children. In addition to

antigen-specific B cell receptors, transitional B cells express several PRRs in addition to antigen-specific B cell receptors that may play a role in their maturation (19). Transitional B cells may encounter PRR ligands during infection or immunization; however, the response of human transitional B cells to these stimuli remains largely unknown. To date, the effects of only select TLR ligands on transitional B cells have been explored (20-23). To fully understand the adjuvant potential of a range of PRR ligands, it is important to explore their potential effects on the development of immature, peripheral B cells as their maturation and differentiation will produce B cell populations that participate in the germinal center reaction and in germinal-center independent immune responses. The current study explores the effects of PRR ligands, currently being investigated as vaccine adjuvants, on the maturation of human transitional B cells into follicular B cells, as demonstrated by surface phenotype and gene expression changes.

2. Materials and Methods

2.1. B cell isolation

Cryopreserved human cord blood mononuclear cells were obtained from Hemacare (Van Nuys, CA), Lonza (Alpharetta, GA), and STEMCELL Technologies Inc. (Vancouver, BC) and used as a source of transitional B cells. Human tonsils were obtained following routine tonsillectomies from the Kapi'olani Medical Center for Women and Children, Hawai'i Pacific Health System (Honolulu, HI) and were used as a source of mature, Fo B cells. These studies were reviewed and determined not to be human

subjects research by the institutional review boards of the University of Hawai‘i and Hawai‘i Pacific Health. Freshly isolated tonsils were soaked overnight in Hank’s Buffered Saline Solution (HBSS; Thermo Fisher Scientific, Waltham, MA, USA) with 1x penicillin-streptomycin. Tonsils were minced and homogenized through a 40um cell strainer. Tonsil mononuclear cells were isolated by density-gradient centrifugation using Ficoll-Hypaque (GE Healthcare, Chicago, Il, USA). Mononuclear cells were collected at the interphase and washed in 1x HBSS. All cells were resuspended in HBSS with 2% fetal bovine serum for downstream fluorescence-activated cell sorting (FACS) and analysis.

2.2. Antibodies and reagents

The following fluorochrome-conjugated anti-human antibodies and fluorescent dyes were used for flow cytometry analyses: CD19-BV605, CD3-BV421, and CD23-BV421 (BD Biosciences, Franklin Lakes, NJ, USA); IgM-APC (Biolegend, San Diego, CA, USA); and CD19-eVolve 605, CD38-PE-eFluor 610, CD24-APC-eFluor 780, CD21-PerCP-eFluor710, FcRL4-PerCP-eFluor 710, IgD-PE, CD5-APC, 7AAD, Fixable Viability Dye eFluor 506, Propidium Iodide, Rhodamine 123 (Thermo Fisher Scientific). Resiquimod (R848) and glucopyranosyl lipid A (GLA) were obtained from the Infectious Disease Research Institute (Seattle, WA). C12-iE-DAP (iE-DAP), polyinosinic-polycytidylic (Poly I:C), trehalose-6,6-dibehenate (TDB), and CpG ODN 2006 (CpG) were purchased from InvivoGen (San Diego, CA).

2.3. Transitional B cell culture conditions

FACS-sorted transitional B cells ($CD3^-$ $CD19^+$ $Rhodamine123^{hi}$ $CD24^+$ $CD38^+$) were seeded in a 96-well U-bottom plate at 5 x 10^5 cells/mL in RPMI containing 10% FBS and 1x penicillin-streptomycin (Thermo Fisher Scientific). Transitional B cells were cultured in medium supplemented with IL-4 (100ng/mL; PeproTech, Rocky Hill, NJ, USA), R848 (TLR7/8 ligand; 5ng/uL), GLA (TLR4 ligand; 2ng/uL), CpG ODN 2006 (TLR9 ligand; 0.25uM), iE-DAP (NOD1 ligand; 2.22ng/uL), TDB (Mincle ligand; 20ng/uL), Poly(I:C) (TLR3 ligand; 20ng/uL), or anti-IgM/IgG (Jackson ImmunoResearch; 20ug/mL, 10ug/mL, and 2ug/mL). After 2 and 4 days, transitional B cell cultures were analyzed for mature Fo B cell surface markers ($CD19^+$ $R123^{lo}$ $CD23^+$) by flow cytometry. Each experiment was repeated at least 4 times. Representative data and compiled results of replicate experiments are shown.

2.4. Flow cytometric analysis

Flow cytometry sorting and analyses were performed on a FACSAria instrument (BD Biosciences, San Jose, CA) and an Attune NxT instrument, respectively (Thermo Fisher Scientific) at the Cellular and Molecular Immunology Core Facility at the John A. Burns School of Medicine, University of Hawai‘i at Mānoa. Cells were stained with the appropriate antibodies and incubated in the dark at 4°C for 30 minutes. Cells were then washed twice and resuspended in 1x HBSS supplemented with 2% FBS for sorting and analyses. Analyses were performed using FlowJo data analysis software (FlowJo, Ashland, OR).

2.5. RNA Sequencing

Cord blood transitional B cells and tonsil follicular B cells were sorted and total RNA was extracted using the RNeasy Mini kit (Qiagen, Germantown, MD). Total RNA was submitted to the Genomics Shared Resource at the University of Hawaii – Cancer Center for library preparation and sequencing. Total RNA was quantified, and quality checked using the Bioanalyzer RNA 2100 Pico instrument (Agilent, Santa Clara, CA). Libraries were prepared with AmpliSeq for Illumina Transcriptome Human Gene Expression Panel (Illumina, San Diego, CA). Sequencing was performed on a NextSeq 500 (Illumina, San Diego, CA). Raw FASTQ files were transferred to the Bioinformatics core at the John A. Burns School of Medicine, University of Hawai‘i at Manoa for analysis. Raw reads were processed using *CutAdapt* (24) and aligned to human genome (hg38) using *STAR* (25). Gene counts were quantified using *Partek E/M Quantification* (Partek Inc., St. Louis, MO). *DESeq2* (26) was used to analyze differential gene expression and data were visualized using R. Ingenuity Pathway Analysis (Qiagen Bioinformatics, Redwood City, CA) was used for pathway and network analysis.

2.6. Real-time qPCR

Total RNA was reverse-transcribed into cDNA using the cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). For AmpliSeq confirmation and gene expression analysis following transitional B cell stimulation, cDNA was added to SYBR Green Supermix (Bio-Rad Laboratories) and FCER2, PTPN6, ADAM28, RUNX1, RUNX2,

PRDM1, and CD27 primers (Qiagen). All real-time qPCR analyses were run on an Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific). Expression fold-change was calculated using ACTB as the housekeeping gene.

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, San Diego, Ca). Unpaired Student's *t* tests were employed where appropriate. Data are represented as means +/- SEM. Statistical significance was determined as follows: $p > 0.05$ (not significant), $*p \leq 0.05$, or $**p \leq 0.01$ (significant).

3. Results

3.1. Identification of transitional B cell subset from human cord blood and peripheral blood

Transitional B cells are the recent emigrant B cells from the bone marrow. Following successful migration in the periphery to secondary lymphoid organs, transitional B cells differentiate into mature naïve MZ or Fo B cells. In order to study human transitional B cells, we isolated these cells from cord blood, the richest source of human transitional B cells. Three subsets of transitional B cells, found in the $CD19^+$ $CD24^+$ $CD38^+$ population (14, 27), were identified from cord blood by flow cytometry. Since some mature B cells were also found in the $CD24^+$ $CD38^+$ population, rhodamine 123 (R123) retention was used as an additional marker for transitional B cells, which do not express the ABCB1 transporter responsible for efflux of R123 (28). In contrast, mature B cells express

ABCB1 and extrude this dye (29). Approximately 50% of cord blood B cells have a transitional phenotype as defined by bright staining for R123 (Figure 1A) and high levels of CD24 and CD38 expression (Figure 1B). Consistent with the reports of others (30, 31), cord blood $R123^{hi}$ transitional B cells also were characterized by high IgM and IgD expression and low to no expression of CD23 (Fig. 1B). While cord blood $R123^{lo}$ cells also expressed sIgM and sIgD, their expression of these markers was more heterogenous than the $R123^{hi}$ population. Moderate CD21 expression was observed in both $R123^{hi}$ and $R123^{lo}$ B cells.

$CD24^{hi}$ $CD38^{hi}$ transitional B cells are also found in human peripheral blood, however, at a much lower frequency (approximately 4% of total $CD19^{+}$ cells) (Figure 1D). Peripheral transitional B cells were more heterogeneous in their expression of the various cell surface markers than $R123^{hi}$ cord blood transitional B cells, similar to $R123^{lo}$ cord blood B cells (Figure 1B). Naïve (IgD^{+} $CD27^{-}$) cord blood B cells and adult peripheral blood B cells are known to express TLR 1, 2, 4-10, with the highest levels of TLR 7, 9, 10 (12). Expression of these TLRs suggest that cord blood B cells, including transitional B cells, have the potential to respond to TLR agonists. Because we were interested in the ability of PRR ligands to induce maturation of transitional B cells into follicular B cells, we utilized previously accepted markers, CD23 and ABCB1, to define follicular B cell maturation.

3.2. Transitional B cells mature following PRR stimulation

We were interested in determining whether select PRR ligands have the ability to drive the maturation of transitional B cells. Mature B cell subsets found in human secondary lymphoid organs, such as tonsils, express varying levels of surface CD21, CD23, IgD, and IgM (32, 33). CD23 expression has been used as a marker of mature, follicular B cells (34-37). Therefore, we utilized relative CD23 expression to evaluate B cell maturation in transitional B cell cultures.

Preliminary studies of transitional B cells cultured in standard culture medium resulted in a very low level of B cell survival, consistent with the apoptotic fate of most transitional B cells (27). B cell recovery was greatly improved when the anti-apoptotic cytokine IL-4 was added (data not shown). Therefore, IL-4 was included in all transitional B cell cultures unless otherwise noted.

To evaluate transitional B cell maturation, we stimulated highly purified cultures of $CD3^-$ $CD19^+$ $R123^{hi}$ $CD24^+$ $CD38^+$ transitional B cells (>95% purity) for 48- and 96-hours with PRR ligands and IL-4. Cell surface marker expression levels were the same for both 48- and 96-hour cultures, therefore only 48-hour results are reported. A subpopulation of human B cells expressing variable levels of CD23 was recovered after culture of transitional B cells in the presence of IL-4 alone (Figure 3A), including some cells expressing intermediate levels of CD23, probably corresponding to maturing T2 cells, and others expressing high levels of CD23, corresponding to mature, naïve B cells (29).

IL-4 has been found to induce CD23 expression and the maturation of bone marrow derived transitional B cells in mice (38).

The addition of R848, iE-DAP, and CpG to IL-4 containing cultures enhanced the maturation of transitional B cells to $CD23^{hi}$ mature B cells, with very few $CD23^{-}$ cells remaining at 48 h (Figure 3B, D, G). Interestingly, CpG alone (without added IL-4) preserved transitional B cell viability (data not shown) and induced intermediate levels of CD23 expression, however relatively few cells expressed high levels of CD23 (Figure 3H). In cultures containing TDB plus IL-4, transitional B cells retained a mostly CD23 negative phenotype, with a lower percentage of CD23 high or intermediate cells as compared to IL-4 cultures (Figure 3F). $CD23^{hi}$ cells were present at a lower level in these cultures than in IL-4 only cultures. Cultures stimulated with TDB + IL-4 also contained a higher percentage of $CD23^{-}$ $CD21^{+}$ cells than cultures receiving IL-4 alone or IL-4 and other PRR ligands (Figure 6F). Poly I:C and GLA had little effect on the differentiation of transitional B cells relative to IL-4 alone (Figure 3C, E).

This overall pattern of responsiveness was consistent for cord blood transitional B cell samples from five individuals (Figure 3I-K). Transitional B cells stimulated with R848 plus IL-4 and CpG plus IL-4 yielded the highest percentage of $CD23^{hi}$ cells (Figure 3I: R848+IL4 vs. IL4, $p=0.0148$; CpG+IL4 vs. IL4, $p=0.0017$) although CD23 MFIs were not significantly different from IL-4 cultures (Figure 3J). Transitional B cells stimulated with CpG alone resulted in little to no increase in $CD23^{hi}$ cells as compared to IL-4 cultures

and these $CD23^+$ cells displayed a statistically significant lower CD23 MFI than CpG plus IL-4 (P=0.0259; Figure 3I, J). Transitional B cells express CD21 with little to no CD23 prior to stimulation with PRR ligands (Figure 1). Interestingly, the frequency of $CD23^-$ $CD21^+$ cells was higher in cultures stimulated with TDB plus IL-4 (p=0.0476) and CpG alone (not significant) as compared to IL-4 cultures, while other stimulation conditions resulted in an average of less than 3% of $CD19^+$ B cells displaying the $CD23^-$ $CD21^+$ phenotype (Figure 3K). These $CD23^-$ $CD21^+$ cells may correspond to either transitional B cells that have yet to complete B cell maturation or that are differentiating along the marginal zone B cell pathway.

3.3. Transitional B cell stimulation may drive marginal zone precursor population

The previous analysis focused on the expression of CD23 as a marker of B cell maturation, although it serves primarily as an indication of Fo B cell differentiation. The ATP-binding cassette (ABC) B1 transporter has been identified as a marker that discriminates naïve B cells from transitional and all memory B cells (28). The acquisition of ABCB1, as reflected in its ability to extrude the fluorescent dye Rhodamine 123 (R123) from cells, may be used to follow the development of transitional B cells into mature naïve B cells including those that do not fall within the $CD23^+$ population.

Transitional B cells ($CD19^+$ $CD24^+$ $CD38^+$) from cord blood mononuclear cells containing T1, T2, and T3 stages (mainly T1/T2 populations) have been differentiated from mature B cells by their R123, CD24, and CD38 staining intensity (29). Prior to

culture, $R123^{hi}$ transitional B cells were $CD23^{-}$ $CD21^{lo/-}$ with variable IgM and high IgD expression (Figure 4A, red population,). $R123^{lo}$ cord blood B cells correspond to maturing B cells that have begun to efflux R123 via the ABCB1 transporter. These cells were mostly $CD23^{-}$ $CD21^{lo/-}$ IgD^{+} with varying levels of IgM expression, however the $R123^{lo}$ population also contained some IgM^{-} and IgD^{-} cells (blue populations, Figure 4A).

Culture of purified transitional B cells for two days with IL-4, resulted in the emergence of maturing B cell populations that expel varying amounts of the R123 (Figure 4B, blue) as well as a small peak of R123-negative, fully-mature B cells. Maturing B cells have begun to express varying amounts of CD23 with low amounts of CD21. The residual $R123^{hi}$ cells in these cultures were also positive for CD23. After IL-4 culture, $R123^{hi}$, $R123^{lo}$, and $R123^{-}$ populations have increased surface IgM/IgD expression, although a minor subpopulation of $R123^{-}$ mature B cells were negative for both IgM and IgD.

Cells cultured with IL-4 plus R848 generally displayed a higher proportion of fully mature B cells than IL-4 cultures (Figure 4C). These $R123^{-}$ B cells included distinct subpopulations of $CD23^{-}$, $CD23^{int}$, and $CD23^{hi}$ cells, with a majority of cells being $CD23^{hi}$. Similar to IL-4 cultures, the remaining $R123^{hi}$ population was distinct from the original transitional B cells population with respect to CD23, IgM and IgD expression. Interestingly, these remaining $R123^{hi}$ cells were all $CD23^{hi}$, suggesting that CD23 expression increased prior to ABCB1 induction. The $R123^{lo}$ population contains a

majority of IgM^+/IgD^+ and a minority of IgM^-/IgD^- B cells. A summary of transitional B cell phenotypes before and after IL-4/IL-4 + R848 stimulation is shown in Figure 4, inset Table.

Culture with IL-4 or IL-4 plus R848 consistently resulted in subpopulations of $R123^-$ B cells expressing high, intermediate, and no CD23, respectively (Figure 4B-C, orange). An analysis of the R123- cells relative to CD23 expression provided further insight into these phenotypes. R848 + IL-4 induced more $CD23^{hi}$ expression than IL-4 alone. Under both culture conditions, the majority of $CD23^{hi}$ cells were both IgD and IgM high (Supplementary Figure 1). In contrast, the $CD23^-$ population was mostly IgM^{hi} IgD^{hi} with small subpopulations of IgM^+ $IgD^{lo/-}$ and IgM^- IgD^- cells. These $R123^-$ $CD23^-$ B cells are presumably mature naïve B cells that have downregulated their IgM or IgD B cell receptors. Similar to the small population of IgM^+ $IgD^{lo/-}$ we observed, recent literature suggested that another mature B cell population develops in parallel to Fo B cells that are IgM^+ $IgD^{lo/-}$ (39). CD23 has been shown to negatively regulate BCR-mediated activation and is downregulated in isotype switched B cells (40). Additionally, TLR7 and IL-4 stimulation have been shown to induce isotype class switching in activated murine B cells (41). Therefore, the small subpopulation of IgM^- IgD^- cells observed in the $R123^-$ $CD23^-$ population may be isotype switched.

The strong induction of ABCB1 in R848 + IL-4 cultures, along with high CD23 expression indicated that the combination of IL-4R and TLR7/8 signaling were potent

drivers of transitional B cell maturation. While CD23 expression is generally associated with Fo B cells, another population of B cells which express CD23 along with high levels of IgM and IgD are marginal zone precursor cells (42, 43). Our stimulated cells that have effluxed R123 due to the acquisition of ABCB1 resemble the previously described human splenic MZP population (44). However, to identify true MZP cells in our system additional analysis is needed to determine if these cells are capable of further MZ maturation. Thus, it is not possible to distinguish whether maturing transitional B cells under these stimulation conditions represent differentiation along the follicular versus the marginal zone B cell lineages. Interestingly, previous literature has supported the ability of FO B cells to develop into $CD23^{hi}$ $CD21^{+}$ MZP cells and vice versa (42).

3.4. IL-4 is necessary for transitional B cell maturation in the presence of TLR9 ligand and BCR engagement

Transitional B cells are highly sensitive to BCR-mediated apoptosis and this stage of development is thought to represent a checkpoint for deletion of self-reactive clones that migrate to the periphery (27, 45, 46). However, it has been shown that IL-4 can exert a protective effect against BCR-mediated apoptosis for murine bone marrow-derived immature and transitional B cells (28, 38). Additionally, previous data describes BCR engagement as a metabolic activation signal that requires a second signal, either CD40 or PRR stimulus, to maintain survival and prevent apoptosis (47). Since IL-4 alone as well as IL-4 + CpG induced a portion of human cord blood transitional B cells to mature into $CD23^{hi}$ Fo-like B cells (Figure 3G) and CpG may serve as a second signal for B

cells, we next examined whether these stimuli were capable of overcoming the negative effect of BCR engagement on transitional B cells. Transitional B cells were isolated from human cord blood and cultured for 48 hours with IL-4 and/or CpG in the presence or absence of anti-IgM at various concentrations (Figure 5). CpG treatment alone resulted in recovery of a low level of $CD23^+$ B cells (2.76%), however the addition of anti-IgM to CpG-containing cultures resulted in the inhibition the development of $CD23^+$ B cells (Figure 5) although cell viability was maintained (data not shown), indicating CpG may act as a second signal to BCR engagement to promote survival but not maturation of transitional B cells. In contrast, cultures containing IL-4 displayed a trend of dose-dependent increase in frequency of $CD23^+$ cells with anti-IgM treatment (Figure 5). This indicated that IL-4 is capable of protecting transitional B cells from the apoptotic effects of BCR engagement and IL-4 may instead work as a second signal that confirms BCR signaling as an activating signal for B cell maturation. When all three stimuli were provided (Figure 5), the frequency of $CD23^+$ B cells reached maximal levels at even the lowest anti-IgM dose, suggesting a synergistic effect of BCR, IL-4, and TLR9 ligation on transitional B cell maturation.

3.5. Comparison of transitional and follicular B cell transcriptomes

Due to the limited markers associated with follicular B cell maturation and overlapping phenotypes among maturing and mature subsets, it was of interest to define novel markers that distinguish transitional and follicular B cell populations. These markers could be used to confirm whether our conditions for in vitro stimulation with PRR ligands

resulted in the generation of fully mature naive B cells. Transcriptional analysis of murine transitional and follicular B cell populations indicated that each have distinct gene expression profiles (48, 49). However, limited transcriptional data are available for isolated human B cell subsets (50). We performed AmpliSeq analysis on sorted transitional B cells from human cord blood and on follicular B cells from human tonsils to characterize the transcriptome of human transitional and follicular B cells. Transitional B cells ($CD3^-$ $CD19^+$ $CD24^+$ $CD38^+$ $R123^{hi}$) (Figure 2A) and naive follicular B cells ($CD3^-$ $CD19^+$ $CD23^+$ IgD^+) (Figure 2B) were isolated by flow cytometry with >99% purity. 20,000 unique human transcripts were queried and compared between transitional and follicular B cell subsets. RNASeq analysis indicated that 208 transcripts were upregulated, while 618 transcripts were downregulated in the follicular B cell subset compared to the transitional B cell subset (Figure 6A). The top 20 up and down regulated genes in human tonsil follicular B cells compared with human cord blood transitional B cells are shown in Table 2. Select genes differentially expressed in Fo and transitional B cells were confirmed by real-time qPCR (Figure 6B).

The top upregulated genes in Fo B cells included genes associated with cell cycle regulation (CCND1, HJURP), cytoskeletal organization and cell migration (FMNL3, LIMA1), cholesterol biosynthesis and metabolism (DHCR24, TTC39B), nucleotide metabolism (NT5E/CD73), and receptor and vesicle transport (RAB33A, NR3C2). Among the mostly highly-upregulated genes for Fo B cells as compared to transitional B cells were two long intergenic non-coding RNA (lincRNA) genes, AC022182.2 (also

known as ENSG00000254802) and LINC00643. LincRNAs are a subset of long non-coding RNAs (lncRNAs). Expression of several lncRNAs have been reported during B-cell development and maturation (51), and it has been noted that more lncRNAs were differentially expressed between B cell subpopulations than protein-encoding genes (52), making these potential markers for B cell developmental subsets.

Among the genes highly upregulated in transitional B cells were four transcription factors (NR4A3, FOSL2, NR4A2, and TP531NP2), two genes that regulate apoptosis (HRK, NR4A1), genes associated with the extracellular matrix (MMP7, COL1A1), and genes involved in the response to extracellular signals (PDE4A, BSIAP3, LDLRAD4). Another highly-expressed gene in transitional B cells was the cytokine CSF1 which regulates cell migration and cell adhesion. S1PR1, required for migration of immature B cells from bone marrow to blood (53) was highly expressed in both transitional and follicular B cells.

Several of the genes expressed in human transitional and follicular B cells have been previously reported in the B cell literature. In the current study, ADAM28 was upregulated in tonsil Fo B cells relative to cord blood transitional B cells (Table 2). Data from the mouse model has cited a role of ADAM28 in the maturation of murine MZ precursors (MZP) to MZ B cells (43), however its expression in human B cells was not previously reported. ADAM28-positive Fo B cells may be poised to become MZPs, supporting the hypothesized plastic nature of Fo and MZ B cells during B cell development (54).

Pathway analysis examining transcription of genes associated with BCR signaling, PKA signaling, IL-6 signaling, and cyclins & cell cycle regulation found the Fo B cell population to be mostly downregulated in comparison to transitional B cells (Figure 7A-C). Since Fo B cells are resting, naïve B cells that have yet to encounter antigen and receive cognate signals from T cells, they may not be as transcriptionally active as the less mature transitional B cell population. Nevertheless, certain genes of these pathways were more highly expressed in the Fo B cell population including PTPN6 and CD19 involved in BCR signaling and TGFB3 involved in Protein Kinase A signaling (Figure 7A, B). The PTPN6 gene encodes for Shp-1, a well characterized tyrosine phosphatase that acts to negatively regulate BCR phosphorylation activity in resting cells (55). B cell-specific PTPN6-deficient mice have an increased B1a population and a decreased B2 population (56), indicating that PTPN6 may play an important role in the B2 cell fate decision. The observed upregulation of PTPN6/Shp-1 protein tyrosine phosphatase in human FO B cells likely aids in their differentiation and maintenance in a resting state until antigen encounter. The CD19 gene encodes for a transmembrane glycoprotein expressed on all B cells from pro-B to terminal differentiation into plasma cells. CD19 is a critical molecule responsible for proper BCR signaling, adaptor protein recruitment, and BCR-independent signaling via the CD19/CD21 complex (57). We observed higher CD19 expression on tonsil FO B cells as compared to cord blood transitional B cells (Figure 7). The TGFB3 gene encodes for TGF-B3, a pleiotropic cytokine known to suppress murine and human B cell antibody production and proliferation (58). In addition to the role of TGF-B3 in B cell function, resting B cells

secrete TGF-B3 which can expand the regulatory T cell population (59). As Fo B cells are in a resting state, it is likely that they secrete TGF-B3 until antigen encounter or activation via other external stimuli.

B cell maturation and function are highly regulated by members of the TNFR (tumor necrosis factor receptor) superfamily (60). Our transcriptome analysis revealed differential expression of many TNFR superfamily genes (Figure 8). While expression for certain of these genes showed variation between samples, several TNFR superfamily members were consistently up- or down-regulated for all three samples. TNFRSF17, encoding the B cell maturation antigen (BCMA), was one of the consistently upregulated genes in Fo B cells. BCMA is a receptor that binds both BAFF and APRIL, key cytokines involved in normal B cell development. While previously literature indicates that BCMA is not required for normal B cell maturation (61), it is plausible that BCMA expression contributes to Fo B cells development and activation.

Our analysis demonstrates an upregulation of RANKL transcripts on tonsil FO B cells and an upregulation of RANK transcripts on cord blood transitional B cells (Figure 8). The RANKL/RANK (Receptor activator of NF-kB (ligand) signaling pathway plays a key role in bone homeostasis and lymphoid tissue development (62). Membrane-bound and soluble RANKL (TNFSF12) is produced by several lymphoid cells including activated B cells and has been shown to drive osteoclast maturation. RANK (TNFRSF11A) was originally described on dendritic cells, but since then has been described on B cells.

Additionally, mouse studies have shown that RANK-deficiency leads to abnormal B cell development and decreased mature B cell counts in the periphery. Since FO B cells are a recirculating B cell subset, it is likely that their RANKL production is in support of normal bone homeostasis. Transitional B cell RANK transcription supports the notion that B cells require RANK for complete maturation and migration in the periphery.

3.6. CpG + IL-4 and CpG alone induce differential gene expression in transitional B cells

Our AmpliSeq analysis of human cord blood transitional B cells and human tonsil follicular B cells identified several differentially expressed genes. We chose to investigate six genes as potential markers of Fo B cell differentiation: FCER2, PTPN6, ADAM28, PRDM1, and CD27 (Figure 9). FCER2 encodes for CD23, a known follicular B cell marker which was used as a key surface marker for B cell maturation in our studies. In accordance with our previous results, we find that transitional B cells stimulated with CpG + IL-4 express higher levels of FCER2 than CpG-stimulated transitional B cells. PTPN6 expression was found to be upregulated in purified human tonsil Fo B cells as compared to transitional B cells (Figure 7). CpG-stimulated transitional B cells expressed PTPN6, while CpG + IL-4 stimulation did not induce PTPN6 expression. Interestingly, we observed increased ADAM28, PRDM1, and CD27 expression in transitional B cells stimulated with either CpG + IL-4 or CpG alone. Our AmpliSeq results showed that ADAM28 was upregulated in Fo B cells as compared to transitional B cells. Since we observe ADAM28 expression following both stimulation conditions, it is likely that ADAM28 is a general marker of transitional B cell maturation.

PRDM1 and CD27 were upregulated in both of our stimulation conditions. PRDM1 encodes for BLIMP-1, a transcriptional regulator involved in plasma cell differentiation, and CD27 is a marker for both memory B cells and human MZ B cells. PRDM1 and CD27 gene expression in transitional B cells stimulated with CpG + IL-4 or CpG alone suggested that these cells are on a path to terminally differentiate. Additionally, culture conditions that induce CD27 expression may indicate some MZ-like B cell development.

4. Discussion

PRR ligands occur as pathogen components and endogenous host products (21, 23, 63, 64) and are of particular interest as vaccine adjuvants (65). PRR ligands can qualitatively shape the resulting immune response, inducing B cell activation, proliferation and expansion of the antibody repertoire (66-68).

The first part of this study examined the direct effect of PRR ligands on human transitional B cell maturation, focusing on the maturation along the follicular (Fo) pathway. Our study tested the effects of ligands of six PRRs: TLR3, TLR4, TLR7, TLR9, NOD1, and the C-type lectin receptor, Mincle. While there currently is no clear consensus of PRR expression by human B cells, in general, TLR7 and TLR9 have been detected in cord blood and peripheral blood B cells while TLR3 and TLR4 have low to no expression (12, 69-73). NOD1 has been identified on mixed populations of tonsillar B cells (74), however its expression on individual B cell subpopulations and at various

developmental stages has not been determined. Mincle is present in peripheral B cells (75), but its expression in transitional B cells has not been previously investigated.

In a series of experiments using human cord blood as a source of transitional B cells, we found that TLR7, TLR9, or NOD1 ligation in the presence of IL-4 are particularly effective in driving transitional B cell maturation as measured by CD23 expression and ABCB1 activity as measured by R123 extrusion. The majority of transitional B cells stimulated with these PRR ligands matured into $ABCB1^{+}$, $CD23^{hi}$ B cells expressing high levels of sIgM and sIgD. Our results expands upon a previous report that TLR9 ligation can preferentially drive the differentiation of human transitional B cells into mature B cells (20), a capability apparently shared by only a few other PAMPS. Since our in vitro cultures consisted of purified cell populations, our study further demonstrates that human cord blood transitional B cells express functional TLR7, TLR9, and NOD1.

In mice, TLR4 agonists can promote B cell maturation as measured by CD23 and sIgD expression (76). In our in vitro cultures, ligands for TLR3 and TLR4 in the presence of IL-4 did not have a significant effect on human transitional B cell maturation, reflecting a lack of TLR3/TLR4 expression and/or function in these cells. Our data are consistent with other studies suggesting that human B cells express low levels of TLR3 and TLR4 (77). While, it has been reported that IL-4 induces TLR4 expression on human peripheral B cells (78), this did not appear to be the case for cord blood transitional B cells stimulated with IL-4.

The Mincle ligand TDB plus IL-4 inhibited $CD23^+$ B cell maturation while slightly increasing expression of CD21 and FcRL4 (data not shown) in the $CD23^-$ population, suggesting delayed maturation or maturation along the marginal zone pathway by this PRR. The distinct response of transitional B cells to TDB supports the concept that differentiation induced by Mincle may involve a signaling pathway not shared with TLR7, TLR9 or NOD1. Mincle and other C-type lectin receptors signal through FcRγ/Syk and have been shown to induce gene expression independent of other PRR signaling (79). We propose that transitional B cell maturation can be achieved by TLR7, TLR9, and NOD1 stimulation, however, our data could not discriminate whether these maturing B cells were differentiating along the Fo or MZ pathways.

The TLR9 ligand CpG was the only PAMP capable of preserving transitional B cell viability in the absence of IL-4, and it had strikingly different effects on transitional B cell maturation depending on the cytokine environment. In the presence of IL-4, CpG induced B cells to mature and express high levels of CD23 consistent with Fo B cell differentiation. However, in the absence of IL-4, CpG-stimulated the majority of transitional B cell cultures to express intermediate levels of CD23 more consistent with the T2 transitional stage (29). Our results contrast with another study, which reported MZ, rather than Fo, B cell differentiation of transitional B cells stimulated by CpG (20). Since this previous study did not supplement their cultures with IL-4, our results suggest that the presence of specific cytokines in the microenvironment of developing B cells can dramatically influence their differentiation. IL-4 is a pleiotropic cytokine shown to be

necessary to maintain the viability of human transitional B cells in vitro (31, 38). We observed the viability of B cells in our in vitro cultures supplemented with IL-4 to be 15-fold higher than cultures without IL-4 (data not shown). In addition to its anti-apoptotic effect, we found that IL-4 contributes to B cell maturation as measured by CD23 expression and inhibits BCR-induced apoptosis. Our in vitro data are consistent with previous studies in the mouse, which demonstrated that IL-4 protected transitional B cells from apoptosis and supported their maturation to $CD23^{+}$ cells (38).

In addition to CD23, we analyzed R123 retention to track in vitro transitional B cell maturation. Three populations with different levels of R123 were detected following culture with IL-4 with or without PRR ligands: $R123^{hi}$, transitional B cells, $R123^{int}$, maturing B cells, and $R123^{neg}$, fully, mature B cells. $R123^{hi}$ and $R123^{int}$ B cells represented the majority populations found in cord blood B cells prior to culture. At the end of culture, a prominent $R123^{neg}$ population was detected. CD23, CD21, IgM, and IgD expression were analyzed in all three R123 populations to add to our understanding of how surface marker expression changed as transitional B cell maturation progressed. Of note, the CD23 expression and upregulation of surface IgD and IgM were observed prior to R123 extrusion, indicating that these events precede ABCB1 expression in the stepwise progression of B cell maturation. Furthermore, the fully, mature $R123^{neg}$ B cell population contained two subpopulations, a majority population of $R123^{neg}$ $CD23^{hi}$ B cells and a minority population of $R123^{neg}$ $CD23^{neg}$ B cells. IL-4 alone cultures included a larger proportion of $R123^{neg}CD23^{neg}$ B cells than R848 + IL-4 cultures. The

observation that R123^{hi}, R123^{int}, and R123^{neg} populations are mostly IgM^{hi} and IgD^{hi} following both culture conditions, indicated that IL-4 and/or R848 are potent inducers of IgM and IgD expression, hallmarks of mature, naïve B cells. Examination of R123 retention complimented our previous in vitro transitional B cell studies focusing on CD23 acquisition and provided further insight into the step-wise process of B cell maturation.

In the second phase of our studies, we examined the synergistic effect of IL-4 and PRR stimulation as well as B cell receptor stimulation on transitional B cell maturation. Human transitional B cell cultures containing IL-4 and anti-IgM displayed a trend of concentration-dependent increase in CD23^{+} cells. Therefore, in addition to inhibiting BCR-mediated apoptosis in transitional B cells, IL-4 appeared to serve as a second signal along with BCR engagement for promotion of B cell survival and maturation. If the BCR stimulus by itself is considered to mimic central tolerance of transitional B cells, our findings demonstrate that IL-4 can overcome this negative selection, possibly by inhibiting Fas-mediated apoptosis similar to BAFF (80). CpG alone prevented cell death in BCR-stimulated cells but could not fully induce transitional B cell maturation as measured by only partial expression of the CD23^{hi} phenotype (Figure 3H), indicating that TLR9 signaling may be sufficient for cell survival, but insufficient to drive complete transitional B cell maturation.

The third part of this study examined the transcriptomic profiles of human cord blood transitional B cells and tonsil follicular B cells. Our study compared human transitional

and follicular B cell subsets as a means to identify select genes associated with the complete maturation of transitional B cells to a follicular phenotype. Pathway analysis found the Fo B cell population to be transcriptionally quiescent relative to transitional B cells, with the exception of select genes involved in BCR signaling, PKA signaling, and the TNFR superfamily. In contrast, transitional B cells had higher variation of gene expression between samples and higher expression of genes involved in IL-6 and IL-4 signaling, and NF-kB signaling. Further analysis found several genes to be uniquely expressed in either follicular or transitional B cells. Transitional B cells, the precursor population to follicular B cells, contain many differentially expressed genes most likely linked to their stage of development where they are poised for maturation and migration to secondary lymphoid tissue. Harakiri (HRK) has been previously identified as an apoptosis regulatory gene whose gene product interacts with Bcl-2 to induce cell death (81). We observed approximately 6-fold higher HRK expression in transitional B cells than in follicular B cells. HRK may play a role in transitional B cells which are poised to undergo apoptosis in the absence of appropriate survival signals. When peripheral $CD24^{hi}$ $CD38^{hi}$ transitional B cells were compared to the maturing $CD24^{+}$ $CD38^{+}$ and fully mature $CD24^{+}$ $CD38^{-}$, HRK gene expression was highly upregulated (82), consistent with the observations of the current study. Additionally, we observed FOSL2 expression to be preferentially elevated in the transitional B cell population. FOSL2 is a subunit of the AP-1 transcription factor, which regulates B cell proliferation and differentiation via genes essential for early B cell development including Foxo1, Irf4, and Aiolos (83). Previous literature has implicated Aiolos as an important gene in the

follicular or marginal zone B cell fate decision (84). The elevated level of FOSL2 in transitional B cells aligns with the role of these cells as precursors for the mature follicular or marginal zone B cell subsets.

Following transitional B cell migration to secondary lymphoid organs, a fraction will differentiate into follicular B cells. Transcriptome analysis of tonsil follicular B cells identified several genes that may be important in the maturation and maintenance of this population. Previous work in the mouse model has demonstrated that CD19 is necessary for B cell survival and maintenance of the peripheral B cell pool (85). Interestingly, B cell-specific CD19 deficiencies disrupt the Fo and MZ populations, while the transitional B cell population remains intact. The increased CD19 expression found on human tonsil Fo B cells is consistent with the importance of CD19 in mature B cells. Elevated expression of ADAM28 in the Fo B cell population was surprising as previous literature has linked ADAM28 expression to the murine MZ precursor population. However, others have hypothesized that the Fo B cell population may be able to differentiate into MZ B cells. Additionally, our data showing the $R123^{lo}$ maturing B cells express CD23 suggest that a $CD23^{+}$ population may give rise to a mature $R123^{-}$ $CD23^{-}$ MZ population. Therefore, it is plausible that ADAM28 expression on $CD23^{+}$ B cells denotes a population capable of MZ B cell differentiation. The mechanism with which this occurs has yet to be defined.

PTPN6 and TNFRSF17 (BCMA) are both important genes involved in signaling and maintenance of the follicular B cell population. PTPN6 negatively regulates BCR signaling and likely aids in the maintenance of a resting follicular B cell population. BCMA, although previously noted to be indispensable for B cell development, likely contributes to follicular B cell development as a BAFF receptor, a key cytokine in B cell development.

The transcriptional analysis of human transitional and follicular B cell subsets has allowed for the identification of genes unique to their respective subsets. We found HRK and FOSL2 are transitional B cell-specific, while PTPN6, BCMA, and ADAM28 are follicular B cell-specific. Future studies are needed to determine whether these genes are consistent in transitional and follicular B cells from other cell sources. In our brief analysis of gene expression from stimulated transitional B cells, we observe that select genes associated with follicular B cell maturation are expressed. Additional studies are required to establish a complete panel of follicular B cell-associated maturation genes.

As recent bone marrow emigrants, transitional B cells are the earliest circulating B-lineage cells to encounter antigen resulting from infection or vaccination. The effect of the adjuvant constituent of a vaccine on transitional B cells is of particular interest because it can qualitatively alter the resulting immune response, particularly in children who have a higher level of circulating transitional B cells (86) than adults. Previous literature focused on naïve and memory B cell responses to immune modulators like

PRR ligands (66, 71, 87-90). However, transitional B cell maturation during an immune response contributes to the B cell population capable of participating in the adaptive immune response. It is critical that the B cell repertoire, in the context of infection or vaccination, contain clones sufficiently diverse enough to recognize different pathogen variants and PRR stimulation of transitional B cell maturation may broaden the resulting mature B cell repertoire. A dual control system for transitional B cell maturation has been proposed (91) suggesting that BCR/BAFFR signaling cooperate to produce a B cell repertoire with diverse BCR affinities. Our in vitro model suggests that IL-4 and PRR signaling may behave similarly to BAFFR signaling in this dual control system by expanding the B cell repertoire in an antigen-independent manner.

Our current studies indicate that transitional and follicular B cells have unique gene expression patterns and transitional B cell maturation may be influenced by several environmental cues including IL-4, PRR ligands, and BCR engagement. We propose that these signals work concurrently to create a balanced mature B cell repertoire necessary for a robust and efficient adaptive immune response. Further studies are needed to evaluate the impact of PRR- and IL-4-dependent, antigen-independent signaling on the B cell repertoire, as well as to define how PRR ligands, specifically in adjuvant formulations, affect transitional B cell maturation in vivo.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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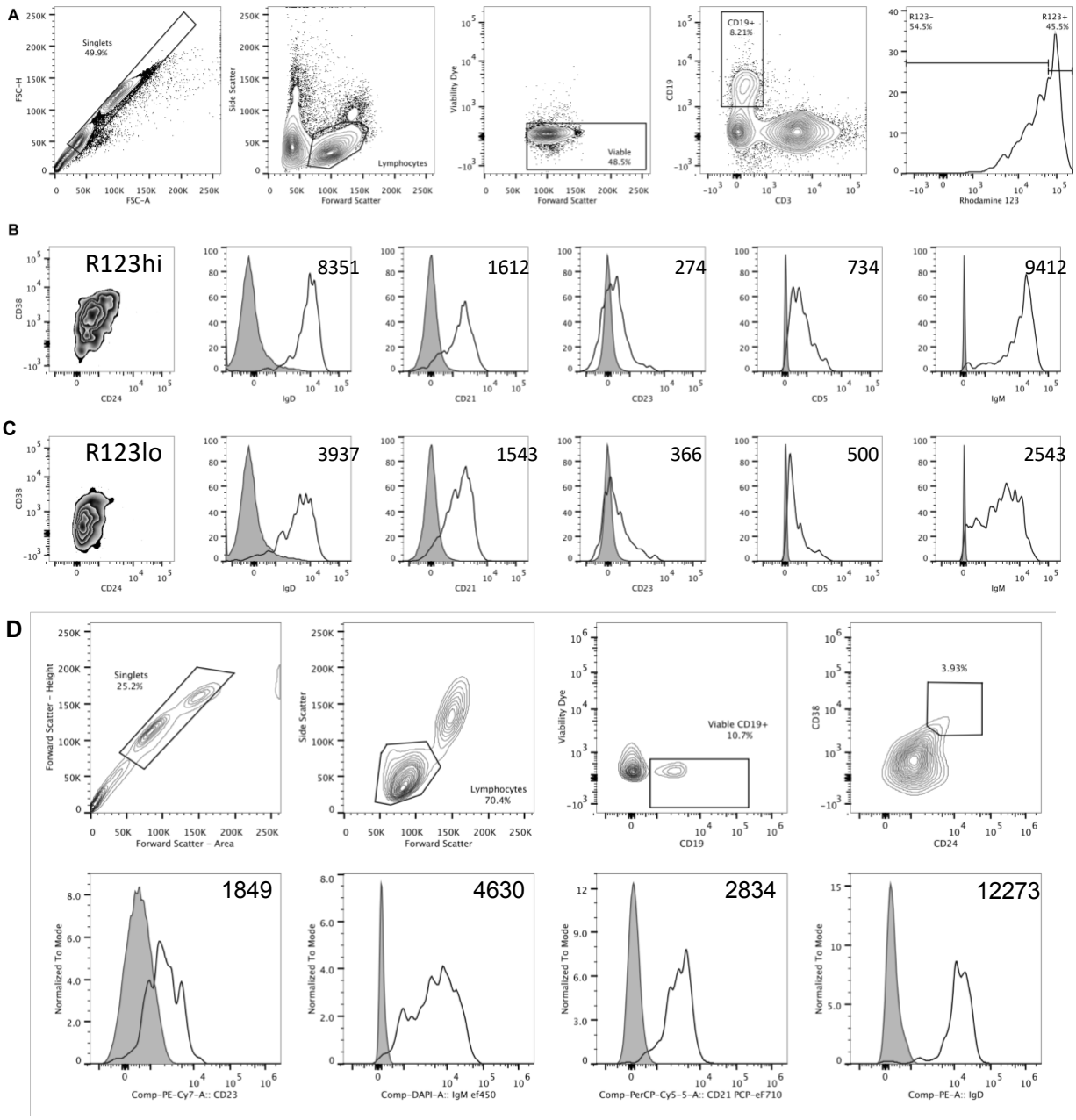


Figure 1. Transitional B cells from human cord blood and peripheral blood mononuclear cells. Cryopreserved cord blood mononuclear cells were thawed and transitional B cells were FACS-sorted based on expression of surface markers, CD19, CD24, CD38 and the presence of Rhodamine 123. Total cells were gated based on lymphocyte morphology, followed by gates on viable $CD19^{+}$ $CD3^{-}$ cells, and finally

Rhodamine 123 fluorescence (A). Expression of IgD, CD21, CD23, CD5, and IgM are shown as histogram plots for high and low Rhodamine 123 expression (B). Freshly isolated PBMCs were labeled with fluorochrome-conjugated antibodies against the surface markers, CD19, CD24, CD38, CD21, CD23, IgD, and IgM. Total cells were gated based on lymphocyte morphology, followed by viable $CD19^{+}$ cells, and $CD38^{hi}$ $CD24^{hi}$ expression. Expression of CD21, CD23, IgD and IgM are shown as histogram plots (shaded histograms are isotype controls) (C).

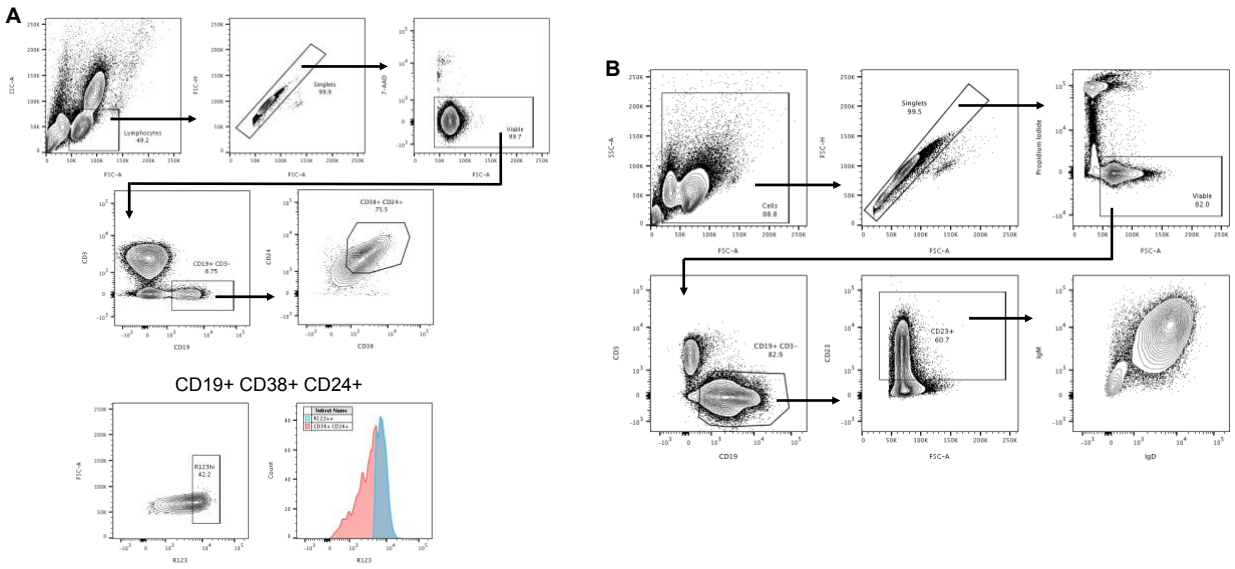


Figure 2. Sorting strategies for human cord blood transitional B cells and human tonsil follicular B cells. Cord blood mononuclear cells were stained with Rhodamine 123 and chased for 3 hours. Following R123 pulse/chase, cells were stained with fluorochrome-conjugated antibodies against CD3, CD19, CD24, and CD38. Transitional B cells were sorted based on high R123 expression following the initial gating strategy (A). Tonsil mononuclear cells were isolated from fresh tonsil tissue via mechanical homogenization and Ficoll density gradient centrifugation. Cell suspensions were stained with fluorochrome-conjugated antibodies against CD3, CD19, CD23, IgD, IgM. Follicular B cells were sorted as $CD3^{-}$ $CD19^{+}$ $CD23^{+}$ IgD^{+} $IgM^{+/-}$.

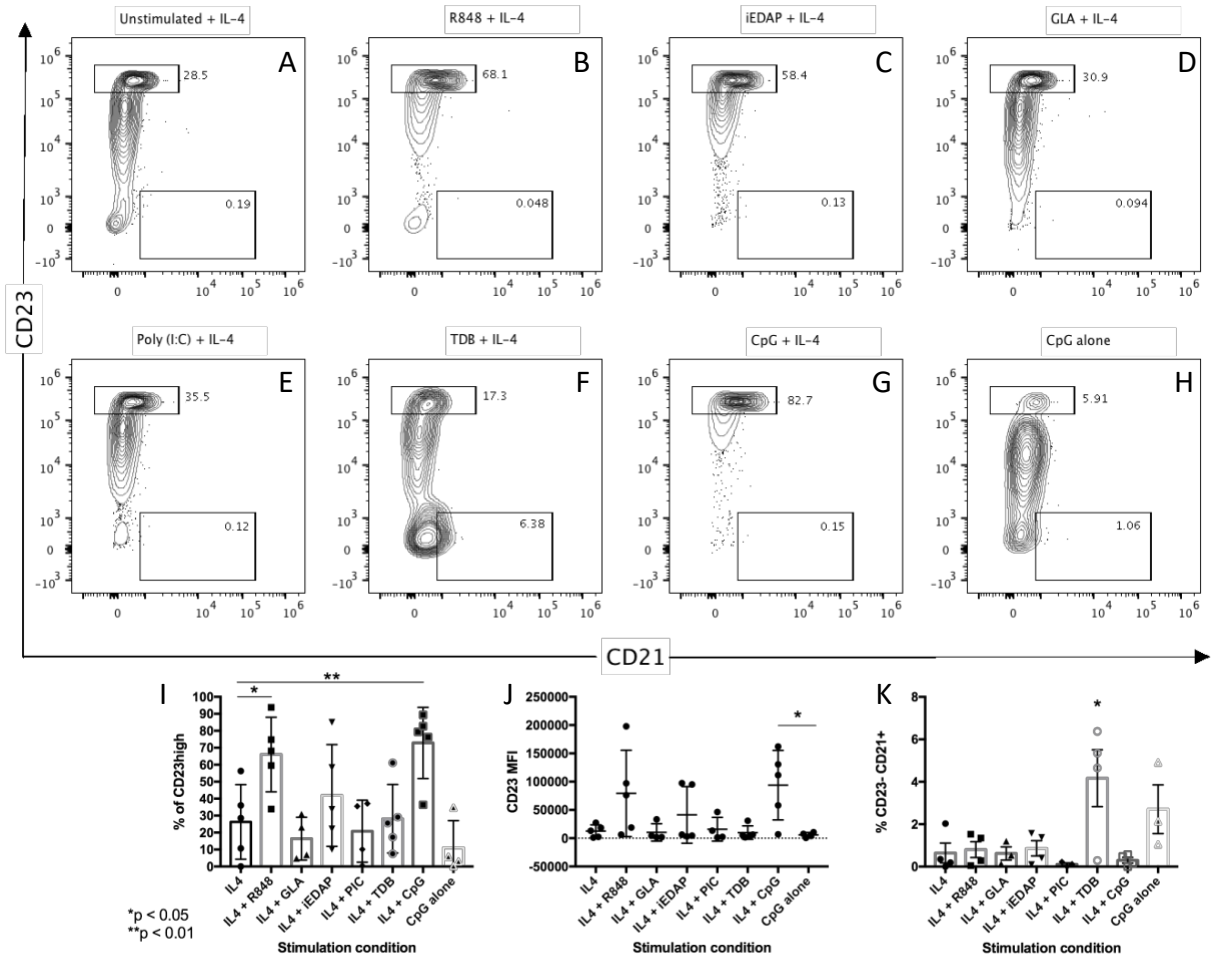


Figure 3. Transitional B cell differentiation into marginal zone-like or follicular-like phenotypes. FACS-sorted transitional B cells were cultured with IL-4 and individual PRR ligands as denoted at $5x10^5$ cells/mL for 2 days. The expression of CD23 and R123 retention was evaluated to determine whether transitional B cells had matured. R123 negative, $CD23^+$ cells were considered mature FO-like B cells. The highest percentage of transitional B cells driven to a Fo-like B cell phenotype were stimulated with R848 (B), iE-DAP (C), and CpG (G) in the presence of IL-4. The proportion of $CD23^+$ cells after stimulation with GLA (D) or Poly(I:C) (E) was comparable to unstimulated cultures (A). A population of $CD23^{hi}$ cells was detected in all cultures with

the highest percentages resulting from R848 and CpG stimulations in the presence of IL-4. A small proportion of cells stimulated with TDB (F) and CpG (H) alone differentiated into a $CD23^{-}$ $CD21^{+}$ population. Data shown are representative of four independent experiments. Statistical significance was determined using Student's t test for plots showing % CD23 high (I), CD23 MFI (J), % $CD23^{-}$ $CD21^{+}$ (K) for combined data * = $p<0.05$, ** = $p<0.01$.

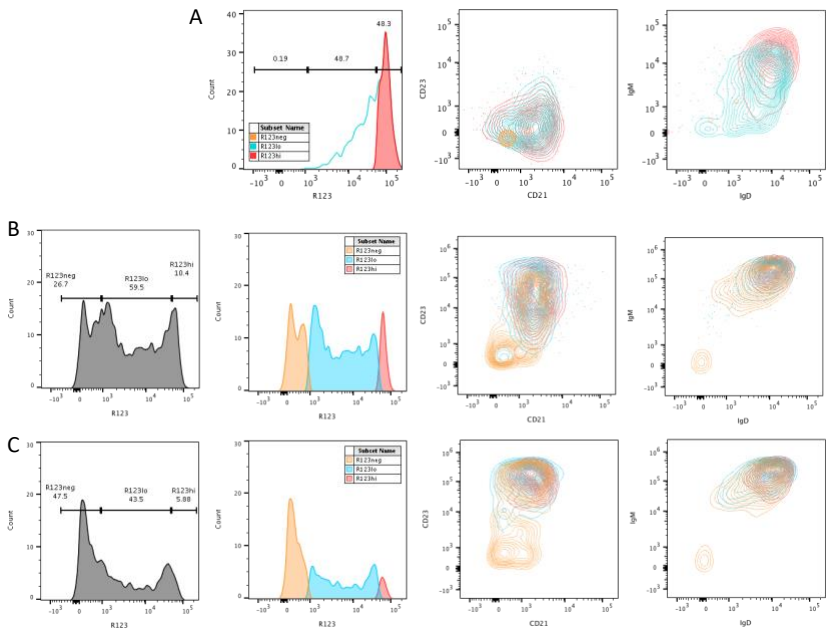


Table 1. Human cord blood transitional B cell population phenotypes before and after IL-4/IL-4+R848 stimulation

	R123^{hi} population	R123^{lo} population	R123^{-} population	
			CD23^{neg} *	CD23^{pos}
Baseline Transitional B cells	ABCB1- IgD+ IgM+/- CD21+/- CD23-	ABCB1lo IgD+ IgM+/- CD21+ CD23-	(very few cells) ABCB1+ IgD- IgM- CD21- CD23-	N/A
Stimulated Transitional B cells	ABCB1- IgDhi IgMhi CD21+ CD23+/-	ABCB1lo IgD+ IgMhi CD21+ CD23+/- **	ABCB1+ IgD+/- IgM+/- CD21+/- CD23-	ABCB1+ IgDhi IgMhi CD21+ CD23+

**CD23 expression in R123^{int} B cells is highest in cells stimulated with both IL-4 and R848

*CD23^{neg} minority mature B cell population is more prominent in IL-4 cultures than in cultures stimulated with both IL-4 and R848

Figure 4. R123 expression highlights three distinct populations of immature transitional, maturing transitional, and mature B cells. Baseline CD23, CD21, and IgD expression on FACS-sorted transitional B cells was similar for R123^{lo} and R123^{hi} cord blood B cells (A). IgM expression on R123^{hi} (red histogram) cells was mostly high, while IgM expression on R123^{lo} (blue histogram) cells was more heterogeneous. FACS-sorted transitional B cells were cultured with IL-4 and IL-4 + R848 at 5x10^{5} cells/mL for 2 days. R123^{hi} (red histogram), R123^{lo} (blue histogram), R123^{-} (orange histogram) expression following stimulation with IL-4 (B) is more varied than IL-4 + R848 stimulated cells' R123 expression (C). Table 1 summarizes the surface phenotypes for R123^{hi}, R123^{lo}, and R123^{-} populations before and after stimulation.

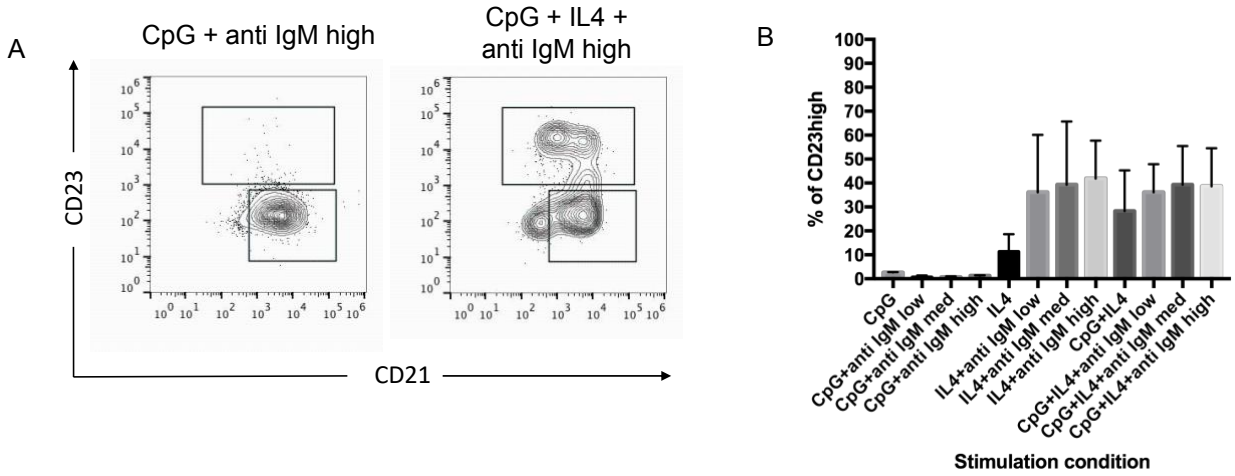


Figure 5. IL-4 is necessary for B cell maturation in the presence of BCR engagement. FACS-sorted transitional B cells were cultured with IL-4 and CpG individually or in combination and with varying concentrations (low-2ug/mL, medium-10ug/mL, high-20ug/mL) of anti-human IgM/IgG at $5x10^5$ cells/mL for 2 days. In the presence of IL-4, the frequency of $CD23^+$ cells increased with BCR engagement in a dose-dependent manner (A). However, when stimulated with CpG plus IL-4, the frequency of $CD23^+$ cells increased with BCR engagement in a dose-independent manner (B). In contrast, in the absence of IL-4, transitional B cells do not mature into $CD23^+$ follicular-like B cells regardless of BCR engagement (C).

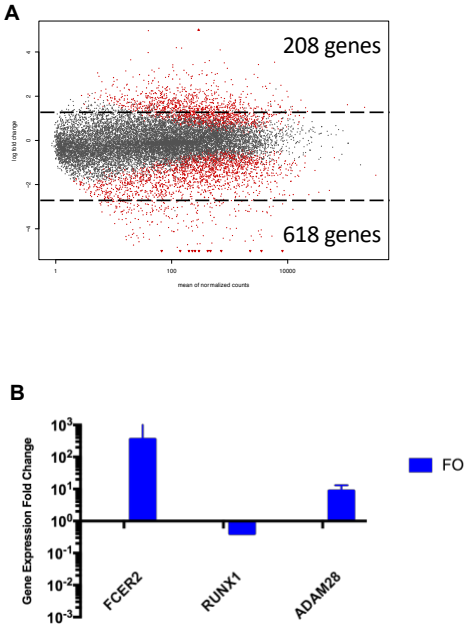


Table 2. Top 20 upregulated genes in tonsil follicular B cells as compared to cord blood transitional B cells.

GENE SYMBOL	DESCRIPTION	log2FoldChange	Pvalue	Padj
AC022182.2	NON-CODING RNA	5.218517081	9.25E-25	1.51E-22
AP002004.1	DYNEIN, AXONEMAL, HEAVY CHAIN 2 PSEUDOGENE	4.964714156	6.69E-11	2.09E-09
DHCR24	CHOLESTEROL BIOSYNTHESIS	4.194323615	2.22E-10	6.23E-09
RAB33A	Rab FAMILY (GTPASE SUPERFAMILY); POSSIBLE VESICLE TRANSPORT	4.178536257	2.41E-17	1.95E-15
GBP1	GUANYLATE BINDING PROTEIN; INDUCED BY IFN	4.096794862	1.12E-20	1.25E-18
LINC00643	LONG INTERGENIC NON-PROTEIN CODING RNA 643	3.977274157	1.07E-12	4.54E-11
CXorf57	RPA1 RELATED SINGLE STRANDED DNA BINDING PROTEIN, X-LINKED	3.645561721	5.99E-10	1.56E-08
MATK	TYROSINE KINASE; INHIBITOR OF SRC FAMILY KINASES	3.608789998	1.61E-29	4.10E-27
CR2	CD21; COMPLEMENT RECEPTOR 2	3.539663418	3.38E-26	6.28E-24
RTP4	RECEPTOR TRANSPORTER PROTEIN 4	3.528108116	6.47E-36	2.84E-33
FMNL3	FORMIN HOMOLOGY DOMAIN; UBIQUITOUS IN LN	3.506524256	1.30E-57	3.43E-54
CHI3L2	CHITINASE 3 LIKE 2	3.432957116	6.74E-12	2.42E-10
HJURP	HOLLIDAY JUNCTION RECOGNITION PROTEIN	3.43167608	7.68E-13	3.39E-11
TTC39B	TETRATRICOPEPTIDE REPEAT DOMAIN 39B	3.418846333	5.21E-12	1.90E-10
CCND1	CYCLIN D1	3.314808877	2.91E-16	2.10E-14
NR3C2	NUCLEAR RECEPTOR SUBFAMILY 3 GROUP C MEMBER 2	3.262200262	8.82E-11	2.74E-09
IVD	ISOVALERYL-COA-DEHYDROGENASE	3.26111869	1.99E-37	1.01E-34
LIMA1	LIM DOMAIN AND ACTIN BINDING 1	3.18135286	5.98E-14	3.08E-12
ADAM28	ADAM METALLOPEPTIDASE DOMAIN 28; NOTCH CELL-CELL INTERACTIONS	3.173104951	3.69E-21	4.38E-19
NT5E	5'-NUCLEOTIDASE ECTO; DETERMINANT OF LYMPHOCYTE DIFFERENTIATION	3.151461426	9.45E-11	2.91E-09

Top 20 downregulated genes in tonsil follicular B cells as compared to cord blood transitional B cells.

NR4A3	NUCLEAR RECEPTOR SUBFAMILY 4 GROUP A MEMBER 3; TRANSCRIPTIONAL ACTIVATOR	-8.088718444	9.24E-133	1.46E-128
LMNA	LAMIN A/C	-7.708745036	1.19E-60	6.27E-57
FOSL2	FOS LIKE 2, AP-1 TRANSCRIPTION FACTOR SUBUNIT; REGULATORS OF DIFFERENTIATION	-6.839376436	2.30E-28	4.91E-26
HRK	HARAKIRI, BCL2 INTERACTING PROTEIN	-6.198552912	4.09E-65	3.23E-61
GABARAPL1	GABA TYPE A RECEPTOR ASSOCIATED PROTEIN LIKE 1	-6.113576104	1.79E-49	2.17E-46
NPTX1	NEURONAL PENTRAXIN 1	-5.899527557	1.45E-15	9.56E-14
NR4A1	NUCLEAR RECEPTOR SUBFAMILY 4 GROUP A MEMBER 1	-5.6199481	4.03E-43	3.18E-40
BAIAP3	BAI1 ASSOCIATED PROTEIN 3	-5.571194202	1.21E-34	4.44E-32
CSF1	COLONY STIMULATING FACTOR 1	-5.49456572	7.18E-40	4.73E-37
PDE4A	PHOSPHODIESTERASE 4A	-5.331981408	4.21E-20	4.46E-18
DUSP8	DUAL SPECIFICITY PHOSPHATASE 8; NEGATIVELY REGULATED MAP KINASE SUPERFAMILY	-5.24340287	4.03E-35	1.55E-32
NR4A2	NUCLEAR RECEPTOR SUBFAMILY 4 GROUP A MEMBER 2	-5.098172728	1.35E-34	4.85E-32
YPEL4	YIPPEE LIKE 4	-4.836815941	3.21E-12	1.22E-10
GALNT9	POLYPEPTIDE N-ACETYLGALACTOSAMINYLTRANSFERASE 9	-4.738199381	2.20E-11	7.47E-10
LDLRAD4	LOW DENSITY LIPOPROTEIN RECEPTOR CLASS A DOMAIN CONTAINING 4	-4.716950472	6.73E-25	1.13E-22
TP53INP2	TUMOR PROTEIN P53 INDUCIBLE NUCLEAR PROTEIN 2; TRANSCRIPTIONAL ACTIVATOR	-4.683664939	1.33E-30	3.68E-28
MMP7	MATRIX METALLOPEPTIDASE 7	-4.67389253	9.34E-19	8.78E-17
AL023775.1	INSULIN LIKE GROWTH FACTOR 2 mRNA BINDING PROTEIN 3 PSEUDOGENE	-4.645116462	1.05E-21	1.27E-19
ODF3L1	OUTER DENSE FIBER OF SPERM TAILS 3 LIKE 1	-4.585112285	3.15E-11	1.04E-09
COL1A1	COLLAGEN TYPE I ALPHA 1 CHAIN	-4.556490431	5.06E-23	7.01E-21

Figure 6. Transcriptome analysis of tonsil follicular B cells and cord blood transitional B cells. Mean differences plot (A) showing the gene expression fold change. Statistically significant differences in gene expression between tonsil follicular B cells and cord blood transitional B cells are in red. The top 20 upregulated and downregulated genes in tonsil follicular B cells as compared to cord blood transitional B cells are listed in Table 2. RT-qPCR confirmation of select genes identified from the AmpliSeq analysis (B).

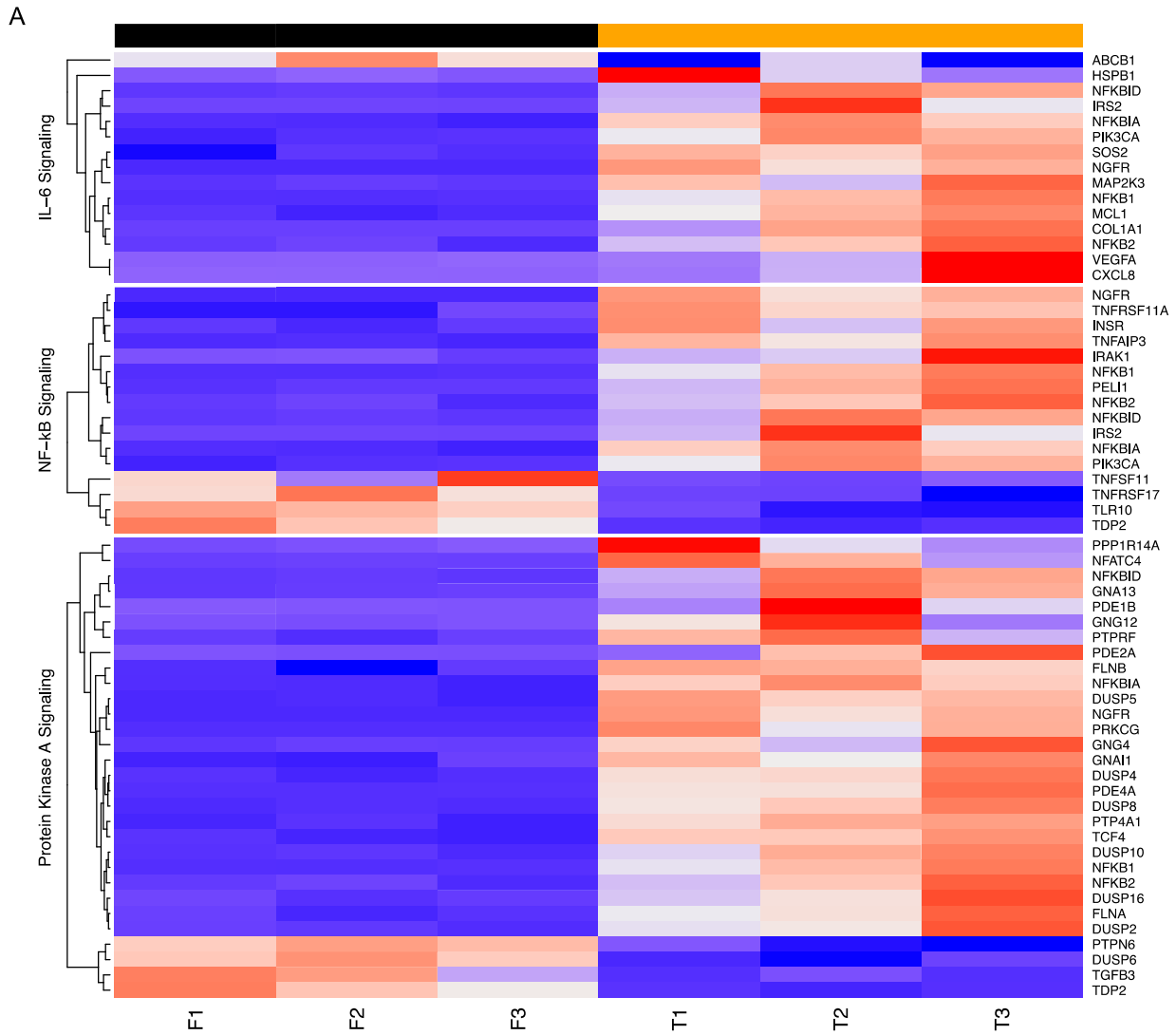
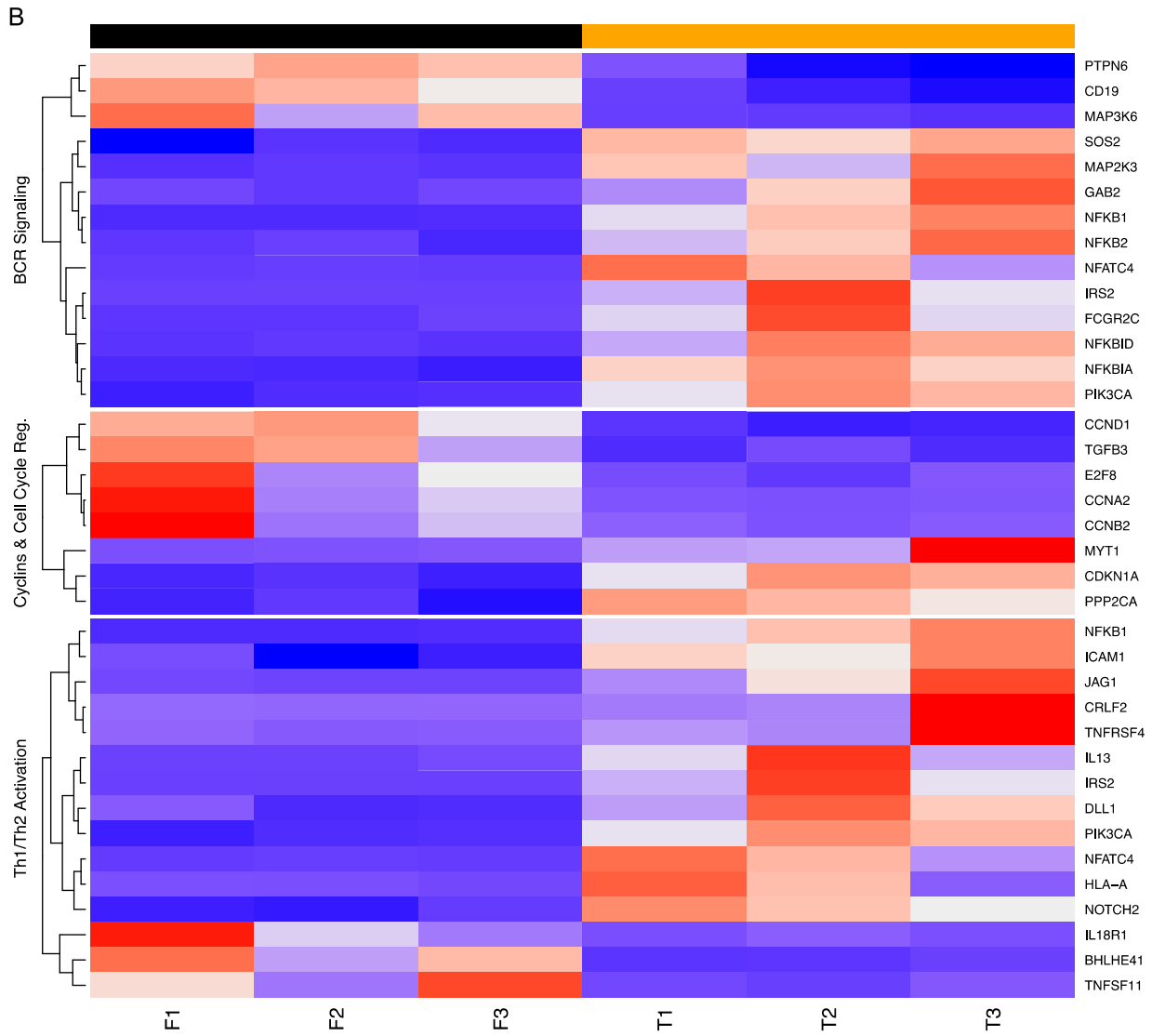


Figure 7. Pathway analysis of tonsil follicular B cells and cord blood transitional B cells. Heatmap display of cell populations from three individuals. Pathways analyzed were IL-6 signaling, NF-kB signaling, PKA signaling (A); BCR signaling, Cyclins & Cell Cycle Regulation, Th1/Th2 activation (B); IL-4 signaling, Notch signaling, PI3K signaling in B lymphocytes (C).



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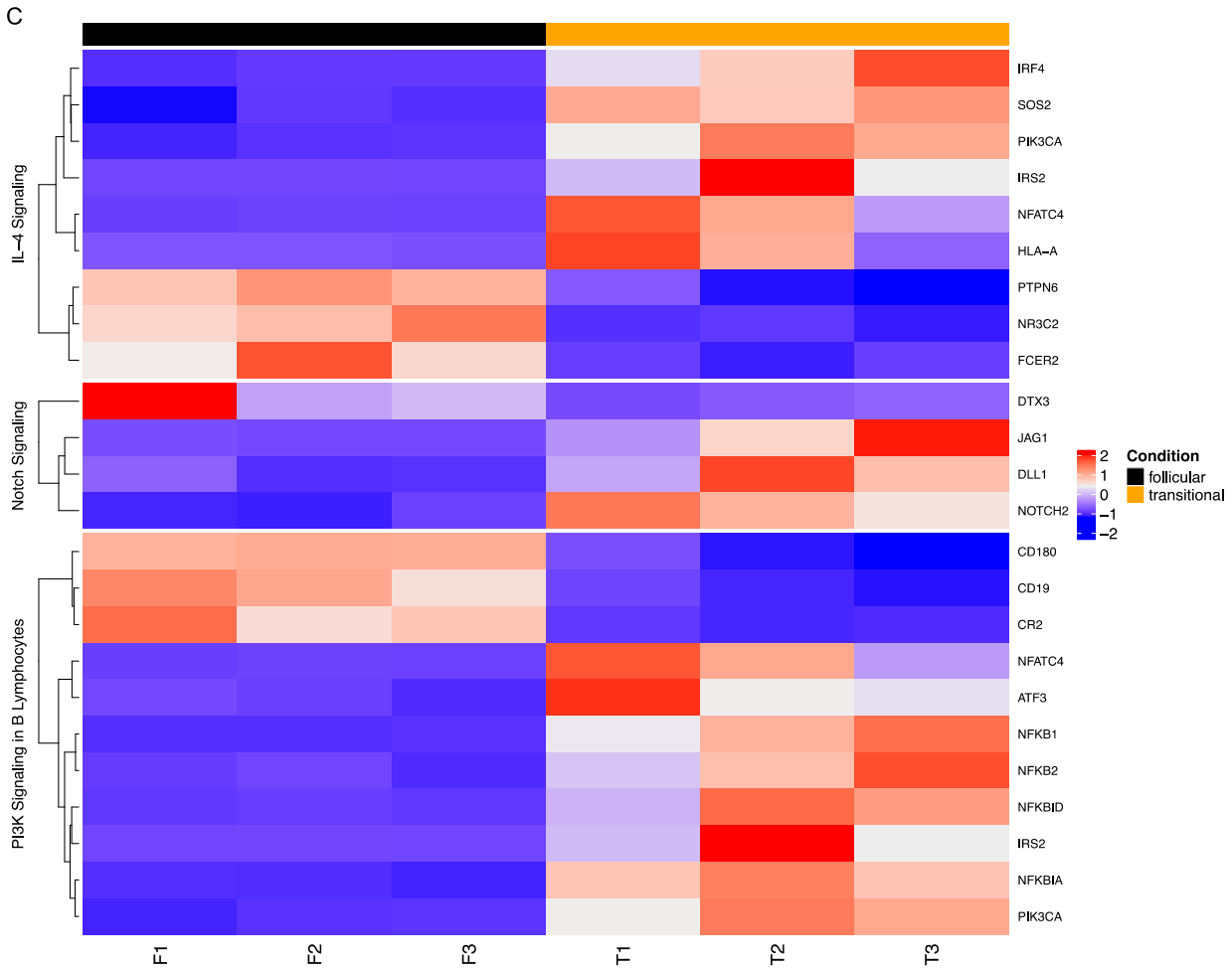

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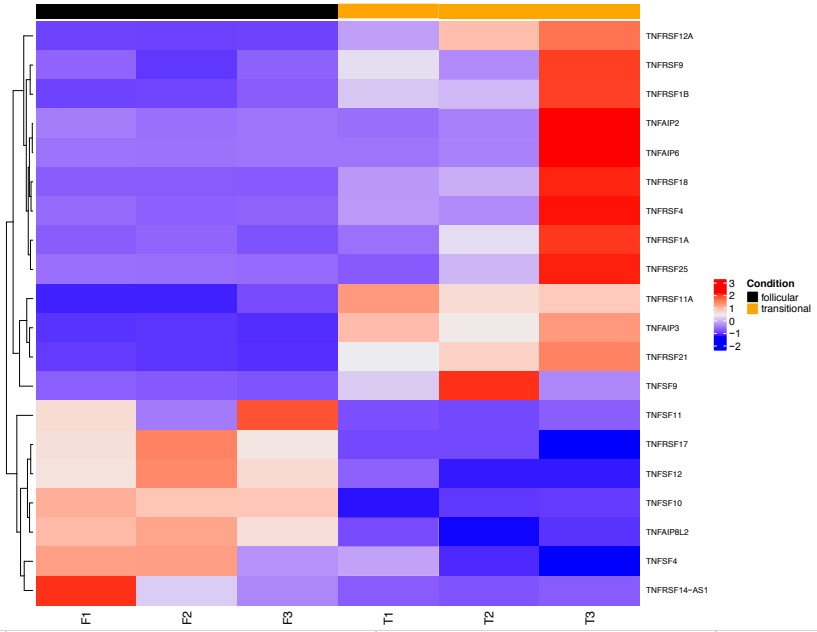


	Gene	Synonyms	Functions	log2FoldChange	pvalue	padj
UPREGULATED IN HUMAN TONSIL FOLLICULAR B CELLS (AS COMPARED TO TRANSITIONAL)	TNFAIP8L2	TIPE2	negative regulator of TLR and TCR function; prevents hyperresponsiveness and maintains homeostasis	2.477275461	6.09E-09	1.29E-07
	TNFSF12	APO3L, DR3LG, TNLG4A, TWEAK	induces apoptosis	2.240205889	4.92E-05	0.00038221
	TNFSF11	CD254, ODF, OPGL, OPTB2, RANKL, TNLG6B, TRANCE, hRANKL2, sOdf	regulation of apoptosis	2.183612636	9.10E-07	1.16E-05
	TNFSF10	APO2L, CD253, TL2, TNLG6A, TRAIL	apoptosis inducer	2.133541862	0.00062152	0.003359
	TNFRSF17	BCM, BCMA, CD269, TNFRSF13A	major receptor for BAFF and APRIL	2.119996653	2.36E-06	2.67E-05
	TNFRSF14-AS1	non-coding RNA		1.362625355	0.00077121	0.00403963
	TNFSF4	CD134L, CD252, GP34, OX40L, TNLG2B, TXGP1	ligand for OX40 (expressed mainly on CD4 T cells); supports Th responses	1.171293841	0.02875562	0.08102038
UPREGULATED IN HUMAN CORD BLOOD TRANSITIONAL B CELLS (AS COMPARED TO FOLLICULAR)	TNFRSF1B	TNFBR, TNFR80, CD120b		-1.246213512	0.0001521	0.00101432
	TNFAIP6	TSG6		-1.503277925	0.00058692	0.00319843
	TNFAIP2	B94, EXOC3L3		-1.580776611	0.00527552	0.02022736
	TNFRSF25	APO3, DR3, GEF720, LARD, PLEKHG5, TNFRSF12, TR3, TRAMP, WSL-1	unkown function in B cells; enhances proliferation in CD4 T cells	-1.682814055	0.0053673	0.0205309
	TNFRSF9	4-1BB, CD137, ILA	expressed on CD40 stimulated B cells; promotes survival via non-canonical NFkB activation in CLL B cells	-1.946836193	0.00123681	0.0060399
	TNFRSF1A	CD120a, FPF, TBP1, TNF-R, p55, p55-R, p60		-1.993570983	0.00026115	0.00161475
	TNFRSF11A	RANK, CD265, FEO, ODFR, OPTB7, TRANCER	receptor activator of NFkB; essential mediator for lymph node development	-2.041163282	0.00210877	0.0094482
	TNFRSF4	OX40, ACT35, CD134, IMD16, TXGP1L	suppresses apoptosis by inducing BCL2	-2.318292277	5.67E-05	0.00042968
	TNFRSF12A	CD266, FN14, TWEAKR		-2.631644263	4.50E-07	6.26E-06
	TNFSF9	4-1BB-L, CD137L, TNLG5A	T cell costimulatory molecule	-2.77999548	1.06E-05	0.00010098
	TNFRSF21	BM-018, CD358, DR6	death receptor 6; induces apoptosis	-4.124865634	1.17E-18	1.08E-16
	TNFAIP3	A20, AISBL, OTUD7C, TNFA1P2	inhibits NFkB activation and TNF-mediated apoptosis	-4.252352143	1.39E-50	1.99E-47
	TNFRSF18	AITR, CD357, GITR, GITR-D	B cell homeostasis	-4.313221404	1.92E-16	1.42E-14

Figure 8. TNFR superfamily transcriptome analysis of tonsil follicular B cells and cord blood transitional B cells. Heatmap display of cell populations from three individuals. Transitional B cells from individual T3 show higher expression of TNFR superfamily genes than T1 and T2.

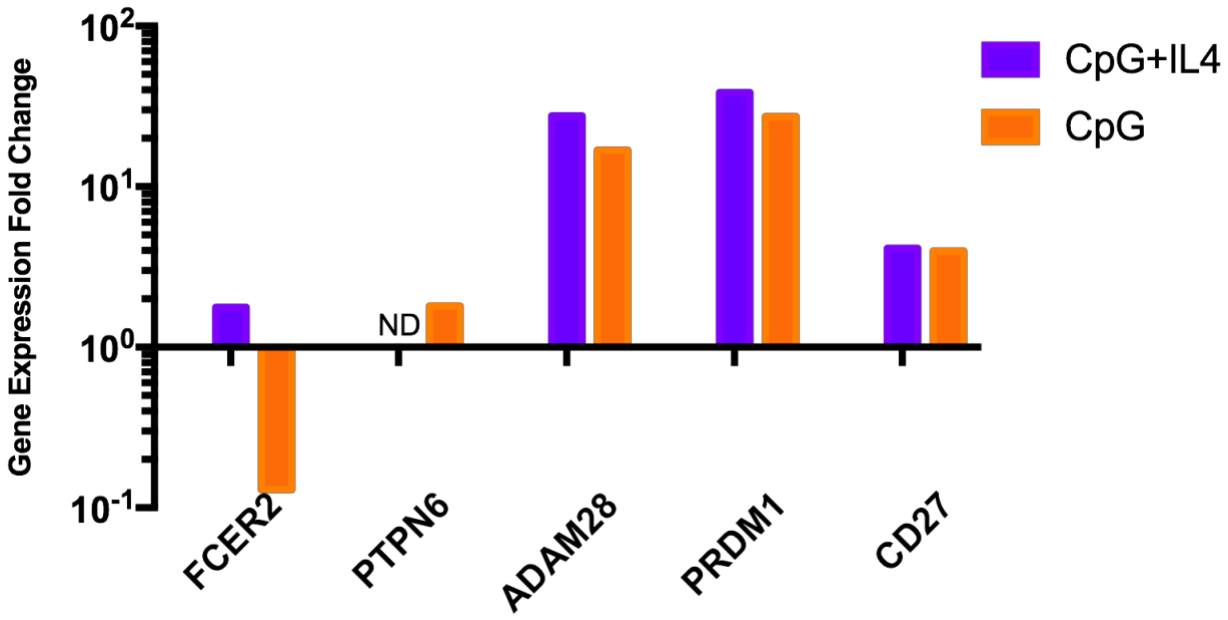
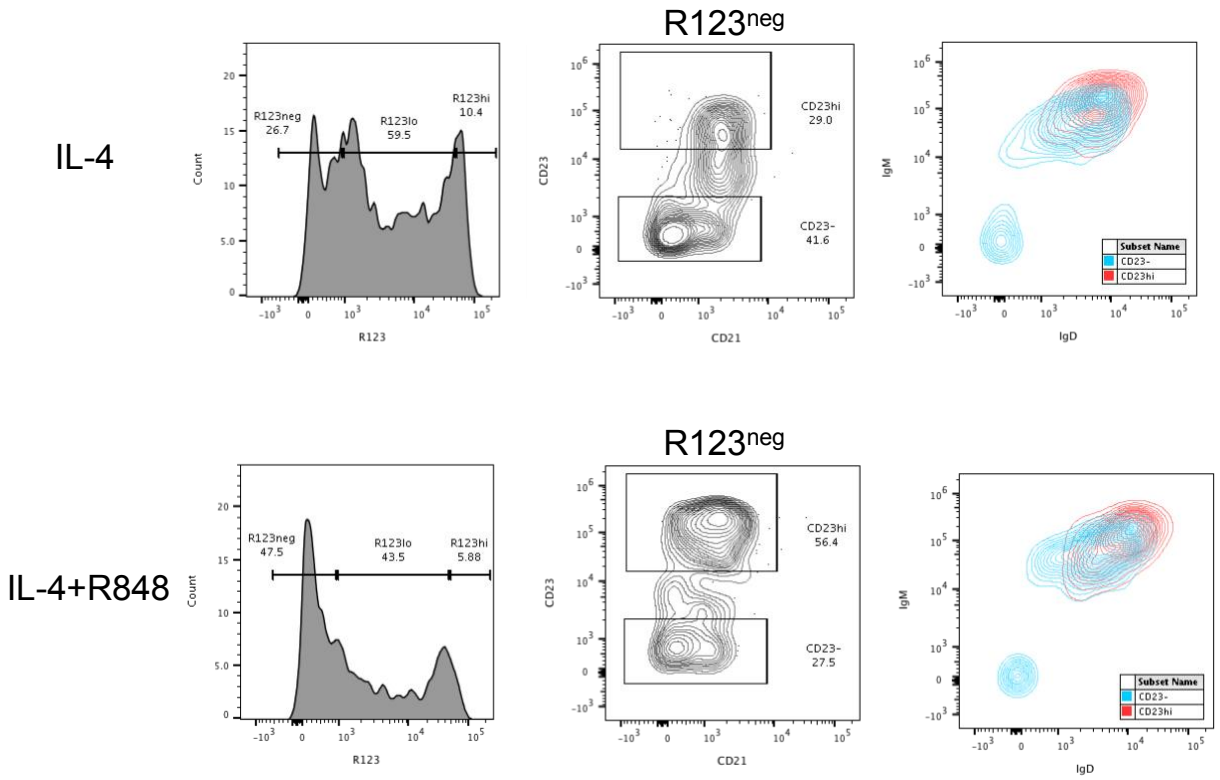


Figure 9. Gene expression analysis of transitional B cells stimulated with CpG + IL-4 or CpG alone. Total RNA from stimulated transitional B cells was isolated and converted to cDNA. FCER2 (CD23), PTPN6 (Shp-1), ADAM28, PRDM1 (Blimp), and CD27 primers with cDNA template and SYBR Green Supermix were used for real-time qPCR reactions. Gene expression fold change was calculated using ACTB as the housekeeping gene and all samples are relative to IL-4-supplemented transitional B cell cultures.



Supplemental Figure 1. Phenotypic analysis of FACS-sorted transitional B cells stimulated with IL-4 or IL-4 + R848. FACS-sorted transitional B cells were cultured with IL-4 and IL-4 + R848 at $5x10^5$ cells/mL for 2 days. IgM and IgD expression on $CD23^+$ and $CD23^-$ cells from the R123 negative population following stimulation with IL-4 and IL-4+R848 were analyzed. IL-4 + R848 stimulation drives more cells to extrude R123 and become R123 negative. The phenotypes of $R123^-$ cells following IL-4 stimulation is mostly $CD23^-$ with some $CD23^+$. $R123^-$ cells following IL-4 + R848 stimulation is mostly $CD23^+$.

CHAPTER 4: ASSESSING THE MARGINAL ZONE B CELL POPULATION

1. Introduction

During normal B cell development, transitional B cells emigrate from the bone marrow and following various stimuli and signaling, differentiate into mature follicular or marginal zone B cells in the spleen. Follicular B cells make up the majority of the mature naïve B cell pool and are a circulating B cell subset capable of recognizing T cell-dependent antigens (1). Following cognate interactions, follicular B cells found the germinal center resulting in high-affinity, antibody-producing plasma cells or memory B cells (2). Marginal zone (MZ) B cells are a subset of mature B cells originally characterized in the marginal zone of mouse spleens. MZ B cells have been implicated as the first line of defense against bloodborne pathogens (3) as well as key players in the rapid IgM response to infection and vaccination (4). While MZ B cells are well-defined in the mouse, they are less defined in the human.

In the mouse, MZ B cells are distinct from follicular and B1 cells with the following phenotype: $CD19^{+}$ IgM^{hi} IgD^{lo} $CD21^{hi}$ $CD23^{-}$ $CD1d^{hi}$ (5). Murine MZ B cells only occupy the marginal zone of the spleen and do not circulate in the periphery. Additionally, murine MZ B cells primarily express non-mutated IgV genes which results in promiscuous antibody reactivity (3, 6). Other characteristics of murine MZ B cells include high expression of Toll-like receptors (TLRs), MHC class II, CD80 and CD86, as well as the ability to respond to T cell-dependent and T cell-independent antigens with low affinity antibody (4, 7-9).

The human splenic marginal zone contains B cells with similar phenotypic and functional characteristics to murine MZ B cells and are considered the human splenic equivalent (5). In humans, the spleen is not the only tissue containing MZ B cell equivalents. Human MZ B cell equivalents are found in various tissues including the subcapsular sinus of lymph nodes, epithelium of tonsillar crypts, and the subepithelial dome of Peyer's patches (10-12). However, with the exception of being uniformly $IgM^{+}CD23^{-/lo}$, these B cells have varying cell surface phenotypes (Table 1).

Cell location	**Cell phenotype**	**References**
Splenic marginal zone	**IgM^{+}** IgD^{-} **$CD23^{-}$** $CD21^{+}$ $CD35^{+}$	(10)
	$FcRL4^{+/-}$ $CD21^{+}$ $CD27^{+}$ $BCL2^{+}$ $CD45RA^{-}$	(13)
Lymph nodes	**IgM^{+}** $CD25^{+}$ $CD5^{-}$ $CD10^{-}$ **$CD23^{-}$** $IgD^{lo/-}$	(11)
Subepithelial layer of tonsils	**IgM^{+}** IgD^{+} **$CD23^{-}$** $CD38^{+/-}$ $CD10^{-}$ $CD44^{+}$	(14)
	$CD19^{+}$ $CD5^{-}$ $CD21^{lo}$ $CD22^{+}$ **$CD23^{lo}$ IgM^{+}** IgD^{lo} $CD27^{+/-}$	(15)
	$FcRL4^{+}$ $CD21^{+/-}$ $CD27^{+/-}$ $BCL2^{+}$ $CD45RA^{+}$	(13)
Subepithelial dome of Peyer's patches	**IgM^{+}** IgD^{-} **$CD23^{-}$** $CD21^{+}$ $CD35^{+}$	(10)

Table 1. Cell surface marker phenotype of human marginal zone B cell equivalents from different locations. Uniform $CD23^{-/lo}$ and IgM^{+} expression is bolded.

This manuscript characterizes the phenotype and gene expression of the $CD23^{-}$ population in human tonsils and investigates the potential for PRR ligands to drive transitional B cells to mature into $CD23^{-}$ B cells. Previous literature suggests that the

CD23$^-$ B cell population contains MZ B cells, IgM memory B cells, and switched memory B cells (16). IgM memory and switched memory B cells have encountered antigen and developed in a GC-dependent or GC-independent manner (17). It is unlikely that our in vitro system can support the development of IgM memory and switched memory B cells. Therefore, transitional B cells that mature in vitro into CD23$^-$ B cells should contain MZ-like naïve B cells.

2. Materials and Methods

2.1. FACS isolation of B cell subsets

Cryopreserved human cord blood mononuclear cells were obtained from Hemacare (Van Nuys, CA), Lonza (Alpharetta, GA), and STEMCELL Technologies Inc. (Vancouver, BC) and used as a source of transitional B cells. Human tonsils were obtained following routine tonsillectomies from the Kapi'olani Medical Center for Women and Children, Hawai'i Pacific Health System (Honolulu, HI) and were used as a source of MZ and FO B cells. These studies were reviewed and determined not to be human subjects research by the institutional review boards of the University of Hawai'i and Hawai'i Pacific Health. Freshly isolated tonsils were soaked overnight in Hank's Buffered Saline Solution (HBSS; Thermo Fisher Scientific, Waltham, MA, USA) with 1x penicillin-streptomycin. Tonsils were minced and homogenized through a 40 um cell strainer. Tonsil mononuclear cells were isolated by density-gradient centrifugation using Ficoll-Hypaque (GE Healthcare, Chicago, Il, USA). Mononuclear cells were collected at the interphase and washed in 1x HBSS. All cells were resuspended in HBSS with 2%

fetal bovine serum for downstream fluorescence-activated cell sorting (FACS) and analysis.

2.2. Antibodies and reagents

The following fluorochrome-conjugated anti-human antibodies and fluorescent dyes were used for flow cytometry analyses: CD19-BV605, CD3-BV421, and CD23-BV421 (BD Biosciences, Franklin Lakes, NJ, USA); IgM-APC (Biolegend, San Diego, CA, USA); CD19-eVolve 605, CD38-PE-eFluor 610, CD24-APC-eFluor 780, CD21-PerCP-eFluor710, FcRL4-PerCP-eFluor 710, IgD-PerCP-eFluor710, CD5-APC, 7AAD, CD86-PE-Cy7, HLA-DR-Alexa Fluor 647, ICOSL-PE-CF594, Propidium Iodide, Rhodamine 123 (Thermo Fisher Scientific). Resiquimod (R848) and glucopyranosyl lipid A (GLA) were obtained from the Infectious Disease Research Institute (Seattle, WA). C12-iE-DAP (iE-DAP), polyinosinic-polycytidylic (Poly I:C), trehalose-6,6-dibehenate (TDB), and CpG ODN 2006 (CpG) were purchased from InvivoGen (San Diego, CA).

2.3. B cell isolation and culture conditions

FACS-sorted transitional B cells ($CD3^-$ $CD19^+$ $Rhodamine123^{hi}$ $CD24^+$ $CD38^+$) were seeded in a 96-well U-bottom plate at 5 x 10^5 cells/mL in RPMI containing 10% FBS and 1x penicillin-streptomycin (Thermo Fisher Scientific). Transitional B cells were cultured in medium supplemented with IL-4 (100ng/mL; PeproTech, Rocky Hill, NJ, USA), R848 (TLR7/8 ligand; 5ng/uL), GLA (TLR4 ligand; 2ng/uL), CpG ODN 2006 (TLR9 ligand; 0.25uM), iE-DAP (NOD1 ligand; 2.22ng/uL), TDB (Mincle ligand; 20ng/uL), or Poly(I:C) (TLR3 ligand; 20ng/uL). After 2 days, B cell cultures were

analyzed for $CD19^+$ $R123^{lo}$ $CD23^-$ IgM^+ $FcRL4^+$ B cell surface markers by flow cytometry and total RNA extracted for downstream real-time quantitative PCR. FACS-sorted marginal zone-like B cells ($CD3^-$ $CD19^+$ $CD23^-$ IgM^+ $FcRL4^+$) and follicular B cells ($CD3^-$ $CD19^+$ $CD23^+$ IgD^+) were immediately lysed for downstream RNA sequencing.

2.4. Flow cytometry analysis

Flow cytometry sorting and analyses were performed on a FACSAria instrument (BD Biosciences, San Jose, CA) and an Attune NxT instrument (Thermo Fisher Scientific) at the Cellular and Molecular Immunology Core Facility at the John A. Burns School of Medicine, University of Hawai‘i at Mānoa. Cells were stained with the appropriate antibodies and incubated in the dark at 4°C for 30 minutes. Cells were then washed twice and resuspended in 1x HBSS supplemented with 2% FBS for sorting and analyses. Analyses were performed using FlowJo data analysis software (FlowJo, Ashland, OR).

2.5. RNA Sequencing

Tonsil follicular ($CD19^+$ $CD23^+$ IgD^+) and marginal zone B cells ($CD19^+$ $CD23^-$ IgM^+ $FcRL4^+$) were sorted by flow cytometry and total RNA was extracted using the RNeasy Mini kit (Qiagen, Germantown, MD). Total RNA was submitted to the Genomics Shared Resource at the University of Hawai‘i Cancer Center for cDNA library preparation and sequencing. Total RNA was quantified, and quality checked using the Bioanalyzer RNA 2100 Pico instrument (Agilent, Santa Clara, CA). Libraries were prepared with 10ng RNA using the AmpliSeq for Illumina Transcriptome Human Gene Expression Panel

(Illumina, San Diego, CA). Sequencing was performed using a NextSeq 500 DNA Sequencer (Illumina, San Diego, CA). Raw FASTQ files were transferred to the Bioinformatics core at the John A. Burns School of Medicine, University of Hawai‘i at Mānoa for gene expression analysis. Raw reads were processed using *CutAdapt* (18) and aligned to human genome (hg38) using *STAR* (19). Gene counts were quantified using *Partek E/M Quantification (Partek Inc., St. Louis, MO)*. *DESeq2 (20)* was used to analyze differential gene expression and data were visualized using R. Ingenuity Pathway Analysis (Qiagen Bioinformatics, Redwood City, CA) was used for pathway and network analysis.

2.6. Gene expression analysis

For real-time quantitative PCR, total RNA was extracted using the RNeasy Mini/Micro kit (Qiagen, Germantown, MD). RNA was reverse-transcribed into cDNA using the cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). For Notch signaling analysis, cDNA was used directly in PrimePCR assays (Notch signaling pathway plates or custom Notch gene plates; Bio-Rad Laboratories). For mature B cell gene expression analysis, cDNA was added to SYBR Green Supermix (Bio-Rad Laboratories) and Btk, Id2, Id3, Asb2, and Tcf3 primers (Qiagen). All real-time qPCR analyses were run on an Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific).

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, San Diego, Ca). Unpaired Student’s *t* tests were employed where appropriate. Data are

represented as means +/- SEM. Statistical significance was determined as follows: $p > 0.05$ (not significant), $*p \leq 0.05$, or $**p \leq 0.01$ (significant).

3. Results

3.1. Tonsil CD23$^-$ B Cell Characterization and Activation State

Tonsils contain CD19$^+$ B cells that express CD21 and moderate to high levels of CD23 as well as cells that are CD21$^{+/-}$ CD23$^-$ (Figure 1). When these two populations are compared, CD23$^+$ cells are IgD$^+$, IgM$^+$ and CD38^{lo}, and are considered to represent Fo B cells (Figure 1A). In contrast, CD23$^-$ cells are either negative or positive for CD21, include both IgD$^+$ and IgD$^-$ cells, are predominantly IgM negative, and include CD38^{lo} as well as a few CD38^{hi} populations (Figure 1A). These CD23$^-$ cells may include MZ B cells as well as IgM memory and switched memory B cells (16). To identify naïve CD23$^-$ B cells, we characterized IgD$^+$ CD23$^-$ tonsil B cells and found that these cells are predominantly IgM$^+$ and CD38$^{lo/-}$ (Figure 1A; bottom row), consistent with the MZ B cell phenotype.

To characterize basal levels of activation, both CD23$^+$ and CD23$^-$ B cells (CD3$^-$ CD19$^+$) were isolated from tonsils and analyzed for CD86 expression (Figure 1B). CD23$^-$ B cells had higher levels of CD86 expression than CD23$^+$ B cells, with the majority of CD23$^-$ B cells being CD86$^+$, similar to previous studies (21). As our CD23$^-$ tonsil B cells included the MZ B cell equivalents, we evaluated our CD23$^-$ population for additional characteristics previously described for MZ populations. As seen in Figure 1B, CD86$^+$ B cells were larger than CD86$^-$ B cells, indicative of more activated cells. While the

proportion of $CD86^+$ cells differed for individual tonsil samples (data not shown), indicating varying proportions of these cells in a resting and activated state, the proportion of $CD86^+$ cells and their CD86 staining intensities were consistently higher for $CD23^-$ B cells than $CD23^+$ B cells. Mouse MZ B cells also have been found to have high levels of CD86 (5). It has been suggested that these cells are in a constant state of activation due to their location between the lymphoid areas and venules, in close proximity to blood-borne antigens (5).

Previous literature has described MZ B cells as first responders to pathogens due to their exposure to infection via blood or mucosal routes (22). Additionally, pathogen and vaccine adjuvant components may drain to lymph nodes and interact with MZ B cells (23). Consequently, it is relevant to determine the effect of PRR ligands on MZ B cell activation. The activation of tonsil $CD23^-$ B cells which contain MZ-like B cells by PRR ligands was measured by upregulation of CD86 expression. FACS-isolated $CD23^-$ B cells were stimulated overnight with the different PRR ligands. We noted that some samples of tonsil $CD23^-$ B cells had relatively high, pre-existing levels of CD86 expression (Figure 1B). Following stimulation, R848, iE-DAP, and CpG moderately increased the percentage of $CD86^+$ $CD23^-$ B cells (Figure 2). In contrast, TDB decreased CD86 expression (Figure 2). The compiled results for five different tonsil samples are provided in Figure 3. Because of the sample-to-sample variation in CD86 expression for unstimulated cultures (Figure 3A,B), results were normalized by calculating the stimulation ratios of PRR-stimulated cultures relative to unstimulated cultures (Figure 3B). Normalized data identified several statistically significant

differences between $CD23^-$ B cells stimulated with various PRR ligands (Figure 3D). Of note, R848-stimulated $CD23^-$ B cell CD86 expression was statistically different from $CD23^-$ B cells stimulated with GLA, iE-DAP, Poly (I:C), and TDB. Additionally, TDB-stimulated $CD23^-$ B cells yielded the lowest frequency of $CD86^+$ cells. While select PRR ligands may induce or maintain CD86 expression on $CD23^-$ B cells, others like TDB may suppress CD86 expression.

MHC class II expression was examined as another potential indicator of B cell activation. PRR ligand-induced upregulation of MHC class II would represent an enhanced ability of B cells to present antigen to T helper cells. Upon isolation from tonsil tissue, $CD23^-$ B cells were characterized by high levels of MHC class II which were unaffected by PRR ligand stimulation (Figure 3E). It has been shown previously that human tonsillar B cells have increased expression of MHC class II following stimulation with TLR2, TLR7/8, and TLR9 ligands (24). However, our results suggest that the $CD23^-$ B cell population from human tonsils already express a high level of MHC class II that is unaffected following PRR stimulation.

3.2. The role of Notch in MZ development

The Notch signaling pathway is important to the development, differentiation, proliferation and survival of mammalian cells (25). In mice, Notch2 signaling is of particular importance during the differentiation of transitional B cells into MZ B cells (26). Previously, we have shown that select PRR ligands can induce CD23 expression on transitional B cells, indicative of Fo maturation (Chapter 3). Transitional B cells

stimulated with R848 (TLR7/8 ligand) + IL4 were found to polarize toward a $CD23^{+}$ Fo-like phenotype, while transitional B cells stimulated with CpG (TLR9 ligand) alone polarized toward a $CD23^{int/-}$ T2/MZ-like phenotype (Chapter 3). However, even for R848 + IL-4, a minority mature B cell population emerged which was $CD23^{-}$. We examined Notch signaling components by real-time qPCR in cultures stimulated under these two conditions. Notch signaling gene expression did not differentiate between B cell populations polarized toward $CD23^{+}$ (Fo-like: R848+IL4 stimulated) versus $CD23^{-}$ (MZ-like: CpG stimulated) phenotypes (Figure 4A). This may have been due to the mixed phenotypes present in stimulated cultures. Nevertheless, one of the genes consistently upregulated in both populations was NOTCH2NL (Figure 4B). The NOTCH2NL gene product is a truncated form of Notch2 that has been implicated in the inhibition of Notch signaling (27). Induction of NOTCH2NL transcription by PRR stimulation may drive transitional B cells to mature via a Notch-independent pathway.

In mice, following Notch2 receptor engagement, the active segment of Notch2 is cleaved and migrates to the nucleus where it binds to the DNA binding protein RBP-J, thus, driving transitional B cell to differentiate into MZ B cells (25, 28). When MINT, a negative regulator or Notch/RBP-J signaling is expressed, it competes for RBP-J binding thereby causing transitional B cells to differentiate into Fo B cells (26). A human homolog of MINT, SPEN, has been identified and may influence the differentiation of human transitional B cells (29). We examined the expression of SPEN in our study and did not observe upregulation of this gene in maturing human B cells (Figure 4A),

suggesting that the alternative NOTCH2NL inhibitor may replace SPEN as a negative regulator of Notch in human transitional B cell development.

While the interplay of PRR ligands and Notch signaling in B cells has not been reported, several groups have examined the interaction of TLRs and Notch signaling in macrophages (25, 30). Macrophages stimulated with TLR ligands were found to upregulate the expression of Notch target genes, indicating cooperation between both pathways. Moreover, cytokines have the ability of modulating this process. Hu et al. found that IFNγ inhibited Notch target gene expression by directly affecting the Notch signaling pathway. Our results raise the possibility that transitional B cell exposure to stimulatory PRR ligands in the presence of IL-4, which like IFNγ utilizes the Jak/STAT pathway, inhibits MZ B cell differentiation in favor of FO B cell differentiation through down-regulation of Notch signaling.

3.3. Mature B cell genes are downregulated following stimulation with TLR7/8 and TLR9 ligands

Since there were no clear differences in the expression of Notch signaling pathway genes using the various PRR ligand stimulating conditions, despite the distinct phenotypes resulting from these stimuli (Figure 6A), we decided to examine the expression of other genes associated with B cell development. The E protein pathway has been found to play a key role in B cell differentiation. BTK, Id2, Id3, Asb2, and TCF3/E47 are E protein pathway genes expressed differentially by MZ and Fo B cells in the mouse (31). Higher levels of expression of Id2 and Asb2 have been reported for

mouse MZ B cells and higher Id3 levels are found in mouse Fo B cells, suggesting that these genes may be differentially expressed in human MZ and Fo B cells and play a role in this cell fate determination. To test this hypothesis, FACS-sorted human tonsil MZ-like B cells ($CD3^-$ $CD19^+$ $CD23^-$ $CD21^{+/-}$ IgM^+) and Fo B cells ($CD3^-$ $CD19^+$ $CD23^+$ $CD21^{+/-}$ IgD^+) were evaluated for expression of B cell differentiation genes (Figure 4C). Consistent with the mouse data, MZ-like B cells expressed higher levels of Id2, Asb2, and TCF3, while Fo B cells expressed higher levels of BTK and Id3 (Figure 4C).

To determine if TLR7/8 and TLR9 ligands drive transitional B cells to express any of these B cell maturation genes, transcripts were analyzed 12 hours post-stimulation with R848 + IL-4 or CpG alone (Figure 4C). In three replicate experiments, all five genes were down regulated in response to TLR7/8 + IL-4 and TLR9 ligation as compared to IL-4 control B cells at this time point (data not shown). However, the degree of downregulation differed between cultures stimulated with R848 + IL-4 and CpG alone. Expression ratios were analyzed to determine the relative gene expression of CpG-stimulated as compared to R848 + IL-4-stimulated transitional B cells. CpG-stimulated cultures expressed slightly higher levels of Asb2 and TCF3 than R848 + IL-4 stimulated cultures, resembling the tonsil MZ-like B cells, and R848 + IL-4 stimulated cultures expressed more BTK than CpG-stimulated cultures, resembling the tonsil Fo-like B cells (Figure 4C). Unexpectedly, Id2, which is expressed at higher levels in MZ B cells in the mouse, was downregulated in CpG-stimulated cultures. Instead, CpG-stimulated cultures had elevated levels of Id3. This suggests that expression of Id2 and Id3 may not be expressed in the same manner by human as reported for murine mature B cells.

3.4. FcRL4 as a marker for tonsil MZ-like B cells

Human MZ B cell equivalents have been shown to express surface IgM, CD27 and contain mutated IgV genes, properties generally associated with memory B cells (5). Due to these similarities between human MZ B cells and memory B cells, controversy exists over whether human MZ B cells are a subset of memory B cells, develop from memory B cells, or are a separate mature B cell population (17, 32).

While CD21 expression is used as a marker of MZ B cells in mice (7), it is expressed by a proportion of both Fo and MZ human B cells (33-35) and therefore cannot be used as a specific indicator of human MZ B cell differentiation. FcRL4 (also known as IRTA1) is selectively expressed on a B cell subset located in the tonsil equivalent of the splenic marginal zone (36). We evaluated surface FcRL4 expression in tonsil B cells to confirm its utility in discriminating MZ B cells from other B cell populations, and in transitional B cell cultures as a marker for in vitro maturation of $CD23^-$ B cells with a MZ-like phenotype.

Human B cell FcRL4 expression was initially examined using tonsillar B cells. Approximately 12% of $CD3^-$ $CD19^+$ $CD23^-$ IgM^+ B cells from the tonsil were found to express FcRL4 (Figure 5). This population was enriched for IgM^+ IgD^+ cells (>75%), characteristic of human tonsil naïve MZ equivalents (12). This is in comparison to the $CD23^+$ IgM^+ IgD^+ human tonsil Fo B cell population which was mostly (98%) FcRL4 negative (Figure 5). Therefore, we hypothesized that FcRL4 expression may allow for better identification of a MZ-like population. Previous studies suggest that MZ B cell

development requires commensal microbiota (37), which contain intrinsic PRR ligands. Additionally, it has been shown that patients deficient in TLR signaling molecules have a diminished IgM^+ IgD^+ $CD27^+$ MZ-like B cell compartment (38), indicative of the role TLRs, and possibly other PRRs, play in MZ-like B cell development. Therefore, we stimulated transitional B cells with select PRR ligands, to determine whether PRR ligands have the potential to induce MZ-like B cell differentiation defined as $R123^{lo/-}$ $CD23^-$ IgM^+ $FcRL4^+$. Upon initiation of culture, less than 1% of transitional B cells are $FcRL4^+$ (Figure 6B). When transitional B cells were stimulated with IL-4 alone or IL-4 along with most PRR ligands, between 5-15% of $CD19^+$ $R123^{lo}$ $CD23^-$ IgM^+ cells acquired the $FcRL4^+$ phenotype (Figure 6C). There was a trend for the highest increase in $FcRL4^+$ cells in cultures stimulated with TDB + IL-4, although this difference was not statistically significant. The induction of FcRL4 expression in cultured transitional B cells supports the interpretation that these cells may be differentiating into MZ-like B cells. However, because stimulation with PRR ligands + IL-4 did not induce more FcRL4 expression than IL-4 alone, PRR signaling may not be a critical factor for MZ B cell maturation, in contrast to our observations on Fo B cell maturation (Chapter 3). It is also possible that other tissue-specific signals and cellular interactions may be required to enhance FcRL4 expression by MZ-like B cells.

3.5. MZ B cell controversy

Recent literature has demonstrated that human MZ B cells have variable surface marker expression and tissue distribution. It is therefore recognized that the human MZ B cell population is heterogeneous but shares three common characteristics: 1) the

ability to recirculate, 2) CD27 expression, and 3) the presence of mutated VDJ genes (39-41). Since these three characteristics are hallmarks of memory B cells, there has been a debate over whether MZ B cells are a subset of memory B cells or represent an independent B cell lineage. This controversy has been reviewed in Chapter 1.

Briefly, utilizing paired blood and tissue samples Aranburu et al. (17) found three types of IgM memory B cells which indicates that the MZ may change with age consisting of innate IgM memory B cells at infancy and GC-experienced IgM memory B cells during adulthood. Therefore, depending on the source and individual's age, the heterogeneous nature of the MZ B cell population may be reflected in transcriptome data.

3.6. Human tonsil MZ-like B cell transcriptome

In light of the controversy over whether marginal zone B cells correspond to a separate B cell lineage or a component of the IgM memory B cell population., several groups have studied the transcriptomes of murine and human B cell populations with marginal zone-like characteristics (42, 43). Kleiman et al. (43) identified murine splenic MZ B cells as $B220^{+}$ IgM^{hi} $CD21^{hi}$ IgD^{-} $CD23^{-}$ $CD24^{int}$ $CD9^{+}$ and compared the transcriptome to four other splenic B cell subsets. Transcriptional analysis revealed that this murine splenic MZ B cell population is enriched for genes that positively regulate NF-kB and control innate immune sensing molecules. Interestingly, a follicular B cell subset (FO-II) shared similar transcriptome expression of innate-like genes with the MZ B cell population, providing support for a plasticity between these populations (44). Descatoire et al. (42) identified human splenic MZ B cells as $CD19^{+}$ IgM^{+} IgD^{+} $CD27^{+}$ $CD24^{+}$

CD38^{lo} and compared its transcriptome to splenic naïve B cells. Microarray analysis revealed that human splenic MZ B cells express select MZ-specific genes related to Notch signaling (DTX1) and transcriptional regulation (SOX7), as well as non-specific markers (CD27, CD300A, TNFSFR13B/TACI). These investigators also identified a subpopulation within naïve B cells that more closely resembled MZ B cells than the rest of the naïve B cell population, suggesting that these cells corresponded to MZ B cell precursors. However, this transcriptome data were from individuals of various ages and we now know that the human marginal zone population is heterogenous and variable with age (17)

Therefore, we decided to analyze the transcriptome of human tonsil MZ equivalents of children. Based on previous literature characterizing the phenotype of tonsil MZ B cell equivalents (12, 36), tonsil CD19^{+} CD23^{-} IgM^{+} FcRL4^{+} MZ B cells were isolated by flow cytometry and their gene expression was compared to tonsil CD19^{+} CD23^{+} IgD^{+} Fo B cells. Using AmpliSeq technology, 20,000 unique human transcripts were queried, and counts were compared between CD23^{-} IgM^{+} FcRL4^{+} and CD23^{+} IgD^{+} B cell subsets. More genes (194 genes) were upregulated in CD19^{+} CD23^{-} IgM^{+} FcRL4^{+} MZ-like B cells as compared to Fo B cells (106 genes) (Figure 7A). The top 10 upregulated and downregulated genes are shown in Figure 7B. Among the highly upregulated genes in MZ B cells were the FCGR2C gene (Fc Fragment of the IgG receptor gene/pseudogene) and TNFSF11 (TNF superfamily member 11 (RANKL)). Although initially described as a pseudogene, FCGR2C has multiple alleles, some of which encode functional gene products (45) that bind to IgG-antigen immune complexes and initiate either inhibitory of activating responses (46) and are associated with

susceptibility to infectious diseases. TNFSF11/RANKL RANKL has been shown to be produced by switched ($CD27^+$ IgD^-) and unswitched ($CD27^+$ IgD^+) memory B cells, the latter potentially also containing MZ B cells (47). CCR1 was upregulated in isolated MZ-like B cells consistent with reports of CCR1 expression on tonsil intraepithelial B cells and lymphocytes in the mantle and marginal zones of follicles in the spleen (48). SIGLEC6 upregulation has not previously been noted in tonsil MZ B cells. However, previous work has shown that Siglec-6, a sialic acid-binding immunoglobulin like lectin, is almost exclusively expressed on peripheral B cells and moderately expressed in spleen tissues (48) indicating its importance for peripheral human B cells, which may include circulating splenic marginal zone B cells.

A distinct set of highly differentially expressed genes for Fo B cells were SPRY1 (Sprouts RTK Signaling Antagonist 1), ADAM23 (metallopeptidase), FCER2 gene encoding CD23, and S1PR1 (sphingosine-1-phosphate receptor 1 (Figure 7). SPRY1, a regulator of receptor tyrosine kinase signaling, has been shown to be involved in the regulation of $CD4^+$ and $CD8^+$ effector responses through its effect on TCR signaling (49) and may serve a similar function in BCR signaling. S1PR1 has been shown to be upregulated in B cells that have reached the mature follicular state (50) and may be required for egress of naïve B cell from lymphoid tissue back into circulation.

The MZ-like tonsil B cell population used in our study was highly enriched for FcRL4. Previous gene expression analysis comparing $FcRL4^+$ and $FcRL4^-$ tonsil B cell populations revealed similar expression profiles to our FACS-isolated tonsil MZ B cells

(51). Ehrhardt et al. identified TNFSF11 (RANKL), RUNX2, and SOX5 as FcRL4^{+} B cell genes because they are expressed exclusively on FcRL4^{+} tonsil B cells as compared to FcRL4^{-} tonsil B cells and other peripheral B cell subsets. Similarly, we observed increased TNFSF11 (RANKL) expression (fold change: -4.4; p-value: <0.001) and RUNX2 (fold change: -2.45; p-value: <0.001) (Figure 7). In addition to the FcRL4^{+} specific gene expression profiles, AICDA and DUSP4 are upregulated in our MZ-like B cells (Figure 7), similar to previous literature (51).

Ingenuity Pathway Analysis comparing tonsil Fo and MZ-like B cell profiles showed differential gene expression in IL-4 signaling, Notch signaling, PI3K signaling, BCR signaling, cell cycle regulators, Th1/Th2 activation markers, IL-6 signaling, NF-kB signaling, and Protein Kinase A signaling pathways (Figure 8A-C). Genes involved in BCR signaling and responsible for Th1/Th2 activation were primarily upregulated in MZ B cells. Alternatively, most genes involved in PI3K signaling were upregulated in Fo B cells. Other signaling pathways like IL-4, IL-6, and NF-kB have differential expression of select genes within the pathway indicating that resting Fo and MZ B cells utilize different genes in the same pathways. These differences may be due to cell-specific metabolism related to Fo B cells being a resting population until encountering antigen and MZ B cells being an activated B cell subset.

Our transcriptome analysis identified several genes associated with a mature tonsil MZ-like B cell population, including RUNX2 and CD27. To determine whether our in vitro cultures induced gene expression similar to our mature MZ-like B cell population, we

isolated RNA from transitional B cells stimulated with CpG + IL-4 or CpG alone. FCER2 (CD23) was analyzed as a positive control as our previous data showed that CpG + IL-4 induces high CD23 expression ($CD23^+$: Fo-like) and CpG alone induces little to no CD23 expression ($CD23^-$: MZ-like) (Chapter 3). We observed an increase in FCER2 expression in CpG + IL-4 stimulated transitional B cells and a decrease in CpG alone stimulated transitional B cells, in agreement with our protein expression data in Chapter 3 (Figure 9). While RUNX2 expression was down-regulated in transitional B cells stimulated with CpG + IL-4, we expected to see an increase in RUNX2 expression in transitional B cells stimulated with CpG alone. We did not observe any RUNX2 expression in CpG-stimulated cultures in our analyses. CD27 was upregulated in both stimulation conditions indicating that both of our in vitro culture conditions drove some cells to differentiate into either memory or MZ-like populations.

4. Discussion

Human MZ B cells are a controversial B cell subset whose phenotype and transcriptome are still under evaluation. The purpose of this study was to evaluate the phenotype of the human tonsil $CD23^-$ B cell population with respect to MZ-like B cell characteristics and to identify other cell surface markers and genes specific to human tonsil $CD23^-$ IgM^+ $FcRL4^+$ MZ-like B cells. Development of a panel of genes that are highly upregulated in MZ B cells and that clearly distinguish them from the rest of the naïve B cell population should enable clearer assessment of the developing MZ-like B cell population in vitro upon stimulation with various PRR ligands.

The first part of this study evaluated the phenotype, activation status, and gene expression of the $CD23^+$ and $CD23^-$ B cell populations. As described in Chapter 3, human tonsil $CD23^+$ B cells displayed a Fo-like phenotype ($CD21^+$ IgD^{hi} IgM^+ $CD38^{lo/-}$). In comparison, human tonsil $CD23^-$ B cells represented a more heterogeneous population, although further analysis of the $CD23^-$ IgD^+ B cells subpopulation displayed a MZ-like phenotype ($CD21^+$ IgM^+ $CD38^{lo/-}$). Therefore, we were interested in further characterizing the MZ-like equivalents within the tonsil $CD23^-$ B cell population. We found that the $CD23^-$ B cell population expressed high levels of CD86 and MHC class II prior to PRR ligand stimulation. Following R848 and CpG stimulation of the $CD23^-$ B cell population increased the frequency of CD86+ cells. TDB decreased the frequency of $CD86^+$ $CD23^-$ cells. Following PRR ligand stimulation, the $CD23^-$ B cell population retained MHC class II expression, regardless of PRR stimulus. These results supported previous reports that the MZ B cell compartment is in a state of constant activation (5). However, the frequency of $CD86^+$ $CD23^-$ B cells may be modified depending on the PRR ligand stimulus.

Notch signaling and B cell-specific maturation genes have been described in the mouse and human model during MZ-like B cell differentiation (42, 52-56). Our results did not find an overall enhancement of Notch signaling genes in cultures stimulated CpG (MZ polarizing) versus R848 + IL-4 (Fo-polarizing). However, we were able to demonstrated that NOTCH2NL, a Notch2 inhibitor, showed a trend of upregulation in transitional B cells in response to both MX- and Fo-polarizing stimuli (TLR7/8 + IL-4 and TLR9). We suspected that the presence of mixed populations following TLR7/8 + IL-4 and TLR9

stimulation may have confounded the gene expression results. Follow-up studies utilizing more highly purified $CD23^-$ IgM^+ IgD^+ tonsil B cells may provide more clear-cut data. B cell-specific maturation genes involved in the E protein pathway (BTK, ID2, ID3, ASB2, TCF3) were expressed in our mature tonsil Fo and MZ-like B cells, in accordance with murine B cell gene expression. Following in vitro stimulation of transitional B cells with R848 + IL-4 or CpG alone, we found that the relative gene expression of BTK, ASB2, and TCF3 corresponded with mature Fo and MZ B cells in the mouse and human.

The second part of this study evaluated whether FcRL4 may be a useful marker to identify MZ-like B cells. The absence of CD23 was useful in isolating a tonsil population containing MZ-like B cells, while FcRL4 was shown to be useful in identifying $CD23^-IgD^+$ IgM^+ naïve MZ-like B cells within this $CD23^-$ B cell population. We attempted to use FcRL4 as a marker of MZ-like B cell differentiation from cord blood transitional B cells in vitro. While transitional B cells were mostly FcRL4-negative, 5-15% of cultured B cells acquired FcRL4 expression after stimulation, which suggests that IL-4 and select PRR ligands may support MZ-like differentiation. However, additional studies are needed to determine if more optimal conditions can be developed to further enhance FcRL4 expression by developing MZ B cells. As an alternative to CD21 and FcRL4 expression, several groups have used CD27 as a marker to identify MZ B cells (16, 42, 57-59). It was also noted in gene expression studies that one of the highly upregulated genes for the FcRL4 population was CD27, suggesting that CD27 and FcRL4 are both expressed in these MZ-like B cells. Conventionally, CD27 has been associated with somatically-

hypermutated memory B cells. Recently, $CD27^+$ IgD^+ IgM^+ and $CD27^+$ IgD^- IgM^+ B cells have been found and characterized in the human splenic marginal zone (34), tonsils and gut-associated lymphoid tissue (16) and many have hypothesized that MZ B cells are a subset of memory B cells. This hypothesis is very controversial and has been reviewed elsewhere (17, 32, 57) and discussed in Chapter 1. CD27 expression in the marginal zone of human spleens does not occur in children younger than two years old (60). From six days to five months of age, most B cells are naïve and IgD-positive. Additionally, this period is associated with an increase in CD27-expressing B cells in the marginal zone. Previous work has shown that CD27-positive B cells are densely located at the follicular periphery, however centrocytes, centroblasts, and plasma cells from these tissues also express CD27 (58). Therefore, although not pursued in this study, CD27 may be useful in identifying MZ B cell equivalents in humans, but only in combination with other markers depending on the tissue of origin. MZ B cells in humans may correspond to a heterogeneous population, with several different phenotypes depending on the individual's age and the tissue in which MZ B cells are located (16, 17).

The final part of this study evaluated and compared the transcriptomes of human tonsil $CD23^-$ IgM^+ $FcRL4^+$ MZ-like B cells and $CD23^+$ IgD^+ Fo B cells. The origin of MZ B cells as a subset of memory B cells or as a distinct lineage arising from transitional B cells or naïve B cells has been disputed. While the origin of MZ B cells remains unclear, there is mounting evidence that the MZ compartment is composed of a heterogeneous population of B cells that is age- and tissue-dependent (16, 17, 32). Comparison of the

transcriptome profiles of human tonsil FO and MZ-like B cells allowed for further characterization of these cells. $CD23^-$ IgM^+ $FcRL4^+$ MZ-like B cells were enriched for IgD^+ cells, indicative of a naïve MZ-like B cell population. Our transcriptome analysis identified several genes specific to human tonsil naïve MZ-like B cells including RUNX2, TBX21, CD27, TNFSF11 (RANKL), and SOX5. CD27, RANKL, RUNX2, and SOX5 have been associated with MZ-like B cells in previous studies (51), while the association of TBX21/Tbet with MZ B cells is a novel finding. Gene expression of transitional B cells induced to mature in vitro by PRR ligand stimuli has been performed for a few of these genes (RUNX2, CD27) and analysis of additional genes are in progress.

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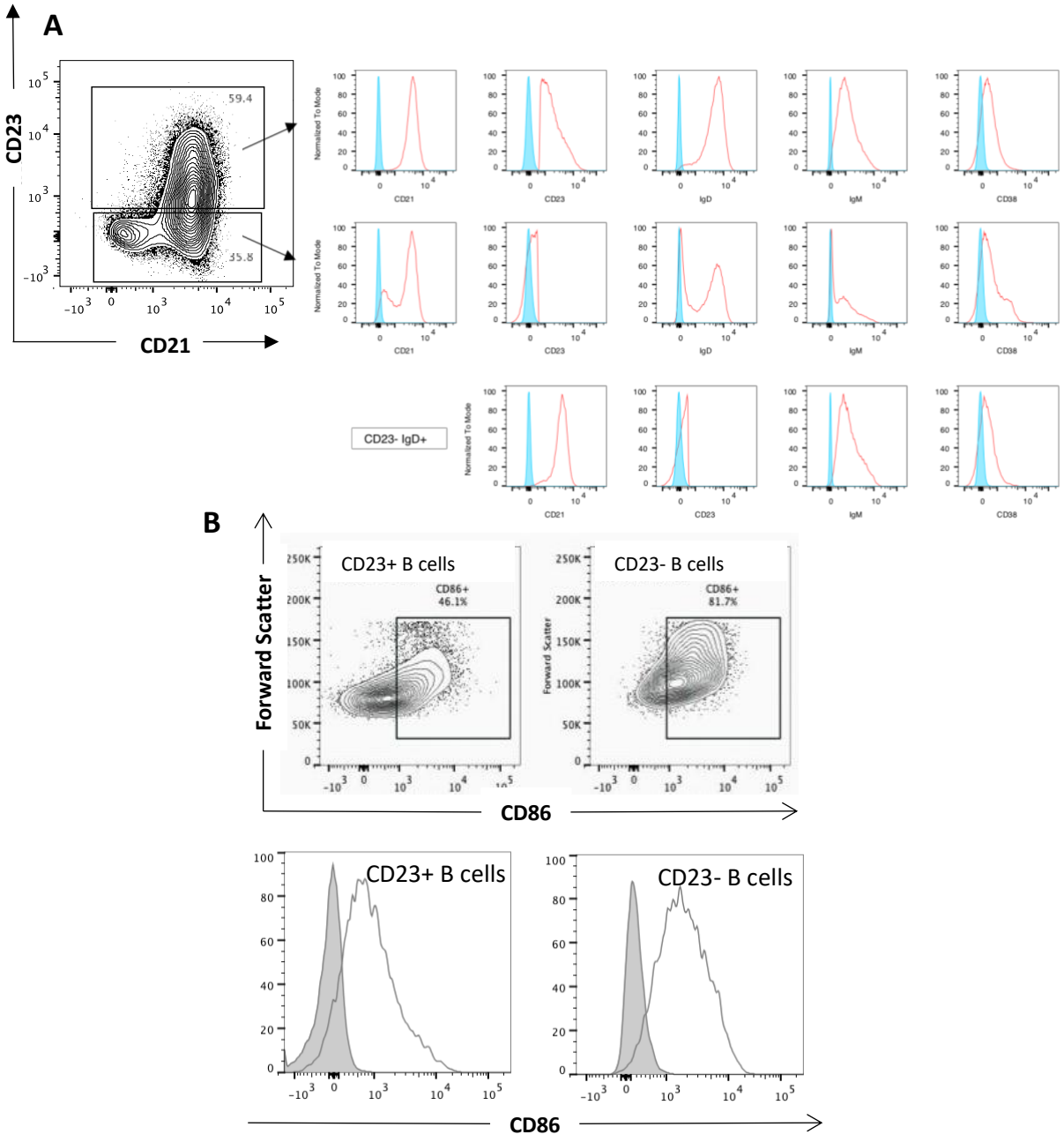


Figure 1. Characterization of tonsil B cells. Histogram plots showing CD21, CD23, IgD, IgM, and CD38 expression of FACS-isolated tonsil $CD3^-$ $CD19^+$ B cells gated on $CD23^+$ B cells and $CD23^-$ B cells, and $CD23^-$ IgD^+ B cells. Isotype control (blue histograms) (A). CD86 expression on $CD23^+$ and $CD23^-$ tonsil B cells (B).

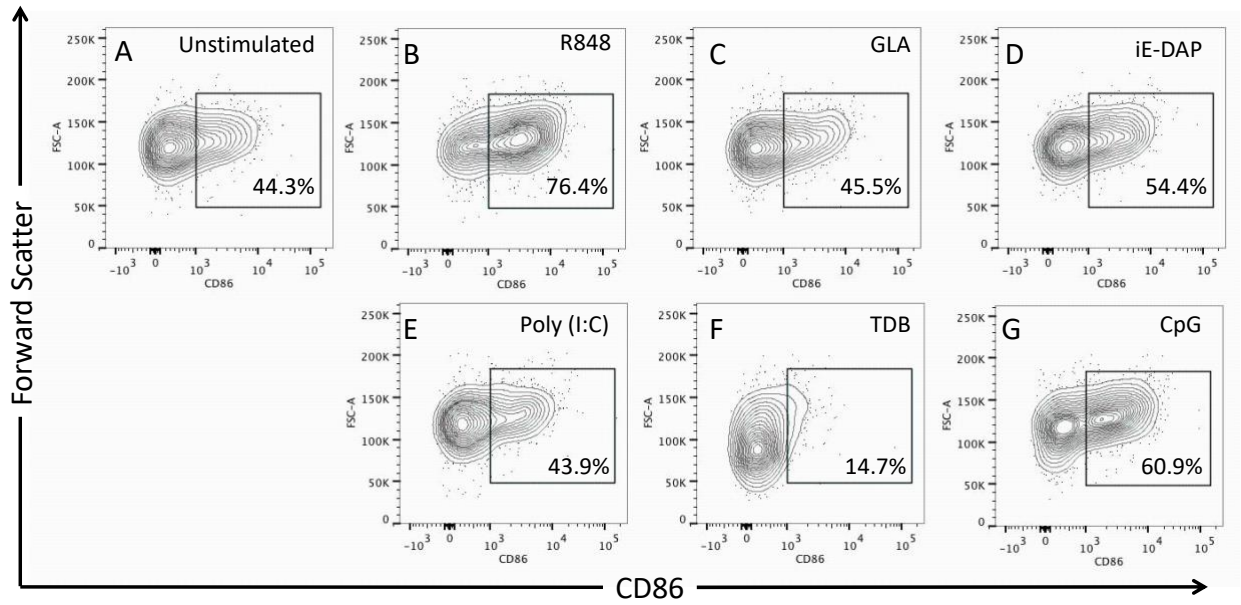


Figure 2. Representative data showing CD86 expression following PRR stimulation with R848, GLA, iE-DAP, Poly (I:C), TDB, and CpG. MZ B cells were FACS-isolated from human tonsil tissue and cultured overnight with PRR ligands. CD86 expression was measured by flow cytometry.

A

	Stimulation						
	Unstimulated	R848	GLA	iEDAP	Poly (I:C)	TDB	CpG
Exp 1	28.7%	44.5%	32.8%	34.5%	29.9%	-	38.6%
Exp 2	44.3%	76.4%	45.5%	54.4%	43.9%	14.7%	60.9%
Exp 3	32.3%	46.4%	30.4%	36.9%	28.5%	10.5%	38.9%
Exp 4	6.95%	13.2%	9.73%	11.1%	6.68%	2.75%	17.8%
Exp 5	10.8%	34.0%	12.4%	17.0%	12.0%	10.4%	11.8%

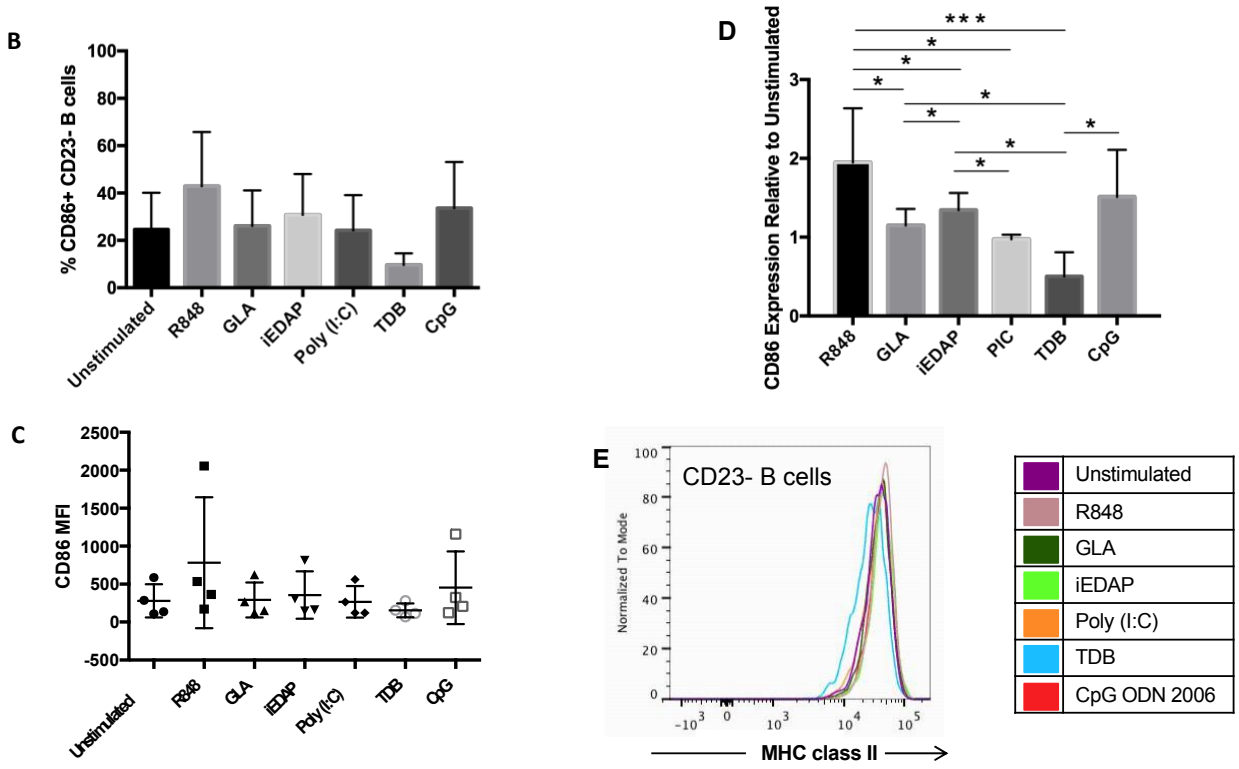


Figure 3. Marginal zone B cell activation following PRR stimulation as measured by CD86 and MHC class II. % CD86+ MZ B cells following PRR stimulation in five individuals (A). Summarized data showing CD86^{+} frequency (B) and CD86 mean fluorescence intensity (MFI) (C) following overnight culture with PRR ligands. Stimulation ratios of % CD86^{+} relative to unstimulated control (D). Representative data showing MHC class II expression on MZ B cells following culture with and without PRR ligands (E).

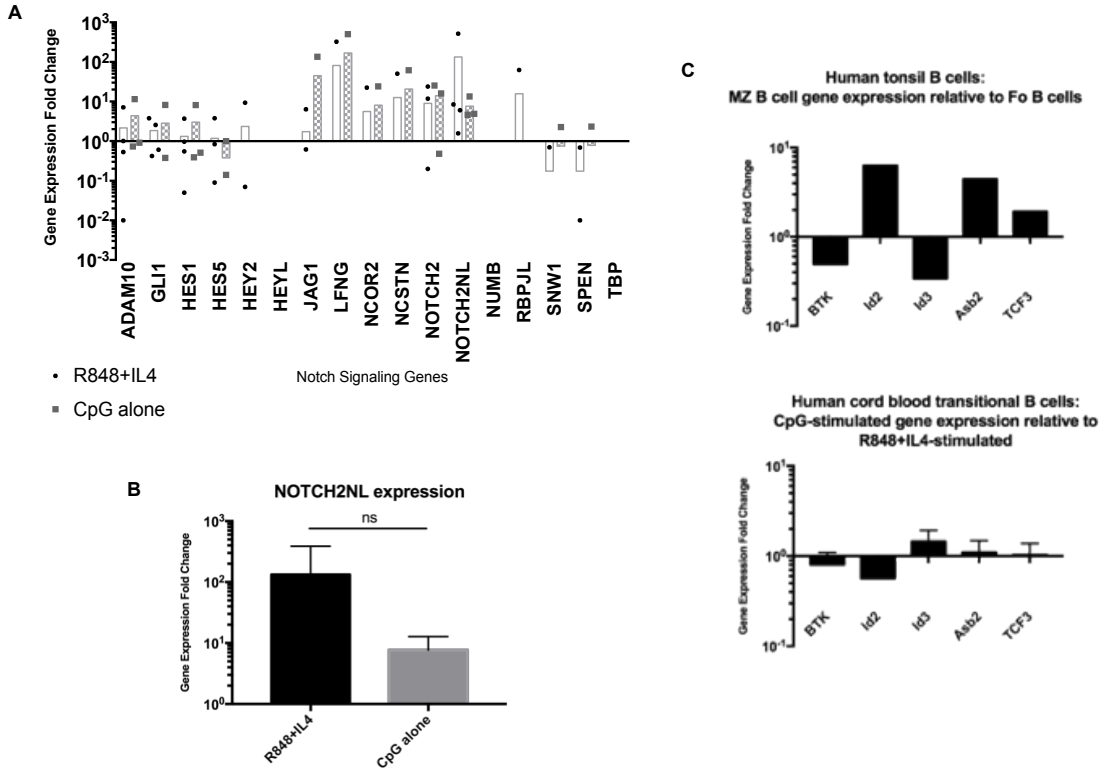


Figure 4. Gene expression analysis of transitional B cells stimulated with R848 + IL4 and CpG alone. Transitional B cells stimulated with IL4 alone, R848 + IL4, and CpG alone were collected 12 hours post-stimulation. RNA was extracted and converted to cDNA for qPCR analysis on Notch Signaling PrimePCR plates. Gene expression fold change is expressed relative to IL4 alone cultures (A). NOTCH2NL expression of transitional B cells stimulated with R848 + IL4 or CpG alone, relative to IL4 alone cultures (B). B cell maturation gene (BTK, ID2, ID3, ASB2, TCF3) expression on tonsil MZ relative to Fo B cells. Human cord blood transitional B cells stimulated with CpG (MZ-like induction) relative to R848 + IL4 (Fo-like induction) (C). Data are representative of at least three experiments.

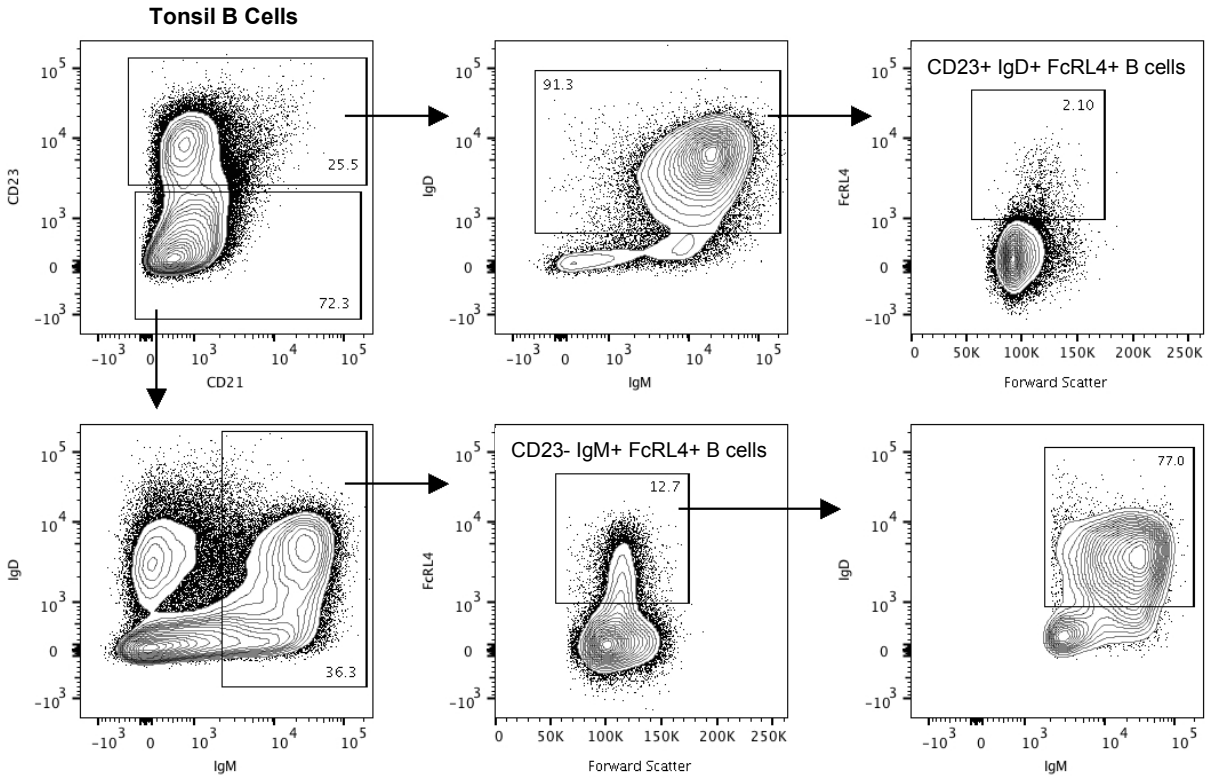


Figure 5. Characterization of FcRL4⁺ tonsil B cells. CD23/CD21 plots show that only 36% of CD23⁻ cells are IgM⁺, with a fraction of those being FcRL4⁺. The majority (77%) of CD23⁻ IgM⁺ FcRL4⁺ tonsil B cells are also IgD⁺, indicative of a naïve population. Most CD23⁺ tonsil B cells are IgD⁺ and only a low frequency are FcRL4⁺.

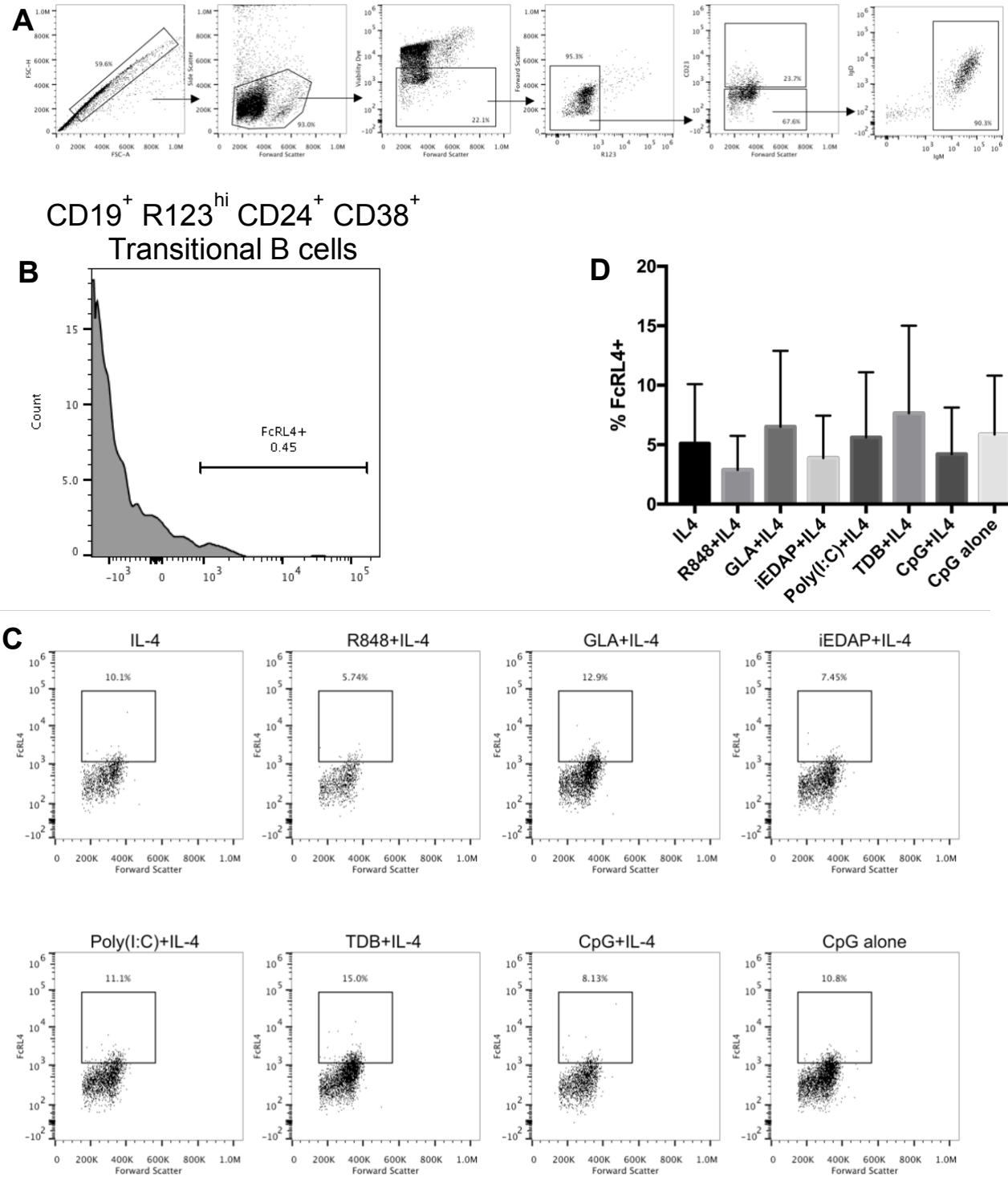
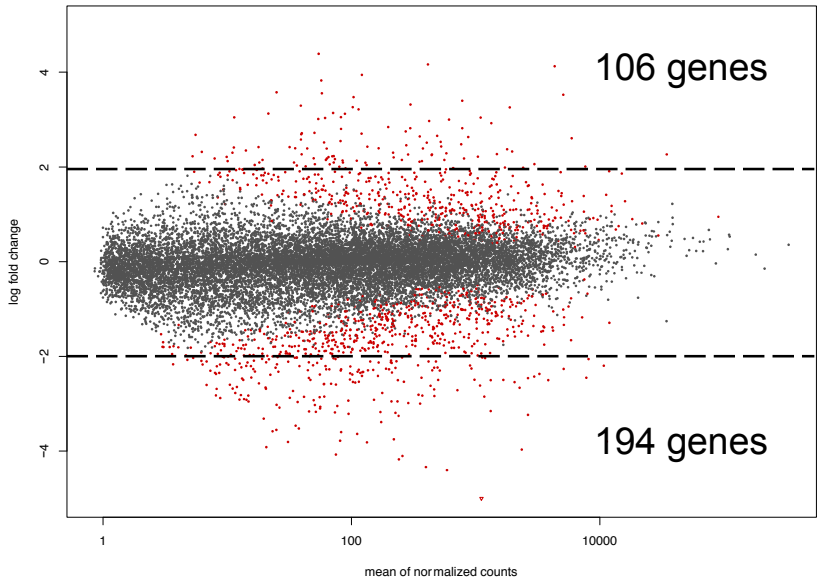


Figure 6. FcRL4 expression following PRR stimulation. Following two-day culture with PRR ligands, cells were gated on single, viable, $R123^{-}$, $CD23^{-}$, IgM^{+} cells (A).

Representative baseline FcRL4 expression on $CD19^{+}$ $R123^{hi}$ $CD24^{+}$ $CD38^{+}$ cord blood transitional B cells (B). Representative data (C) and bar graph (D) summarizing % $FcRL4^{+}$ data for at least two replicate experiments.



	GENE SYMBOL	DESCRIPTION	log2 FoldChange	pvalue	padj
UPREGULATED IN MARGINAL ZONE B CELLS AS COMPARED TO FOLLICULAR B CELLS	FCGR2C	Fc FRAGMENT OF IgG RECEPTOR IIC (GENE/PSEUDOGENE)	-5.3256013	1.97E-31	3.04E-28
	TNFSF11	TNF SUPERFAMILY MEMBER 11	-4.403902	1.36E-27	1.17E-24
	GPR34	G PROTEIN-COUPLED RECEPTOR 34	-4.340436	2.04E-33	3.94E-30
	SIGLEC6	SIALIC ACID BINDING IG LIKE LECTIN 6	-4.1768237	1.53E-28	1.39E-25
	AC068580.4	NOVEL PROTEIN	-4.1054027	1.67E-29	1.85E-26
	MIR4435-2HG	MIR4435-2 HOST GENE	-4.075474	1.73E-17	4.47E-15
	DUSP4	DUAL SPECIFICITY PHOSPHATASE 4	-3.97039	3.28E-24	2.12E-21
	CCR1	C-C MOTIF CHEMOKINE RECEPTOR 1	-3.9182044	4.61E-09	2.82E-07
	CTSW	CATHEPSIN W	-3.8184775	1.03E-23	5.68E-21
	ATP8B4	ATPASE PHOSPHOLIPID TRANSPORTING 8B4 (PUTATIVE)	-3.8097766	9.63E-12	1.10E-09
	RUNX2	RUNX FAMILY TRANSCRIPTION FACTOR 2	-2.4588272	2.80E-11	2.81E-09
	AICDA	ACTIVATION INDUCED CYTIDINE DEAMINASE	-1.4164719	0.00064347	0.00803847
UPREGULATED IN FOLLICULAR B CELLS AS COMPARED TO MARGINAL ZONE B CELLS	SPRY1	SPROUTY RTK SIGNALING ANTAGONIST 1	4.38864963	2.03E-11	2.15E-09
	ADAM23	ADAM METALLOPEPTIDASE DOMAIN 23	4.16427641	8.63E-32	1.48E-28
	FCER2	Fc FRAGMENT OF IgE RECEPTOR II	4.12653057	5.55E-36	1.72E-32
	ENAH	ENAH ACTIN REGULATOR	3.94586063	7.78E-18	2.11E-15
	ST6GALNAC3	ST6 N-ACETYLGALACTOSAMINIDE ALPHA-2,6-SIALYLTRANSFERASE 3	3.82809046	3.18E-15	5.65E-13
	PRICKLE1	PRICKLE PLANAR CELL POLARITY PROTEIN 1	3.57756946	2.24E-11	2.33E-09
	ZNF618	ZINC FINGER PROTEIN 618	3.55618986	2.20E-09	1.42E-07
	TCL1A	T CELL LEUKEMIA/LYMPHOMA 1A	3.52522568	2.10E-16	4.52E-14
	OLMALINC	OLIGODENDROCYTE MATURATION-ASSOCIATED LONG INTERGENIC NON-CODING RNA	3.47306686	3.11E-17	7.51E-15
	S1PR1	SPHINGOSINE-1-PHOSPHATE RECEPTOR 1	3.39918796	6.99E-24	4.16E-21

Figure 7. Transcriptome analysis of tonsil follicular B cells and marginal zone B cells. Mean differences plot (top) showing the gene expression fold change. Statistically significant differences in gene expression between tonsil follicular B cells and marginal zone B cells are in red. The top upregulated and downregulated genes in tonsil follicular B cells as compared to marginal zone B cells are listed.

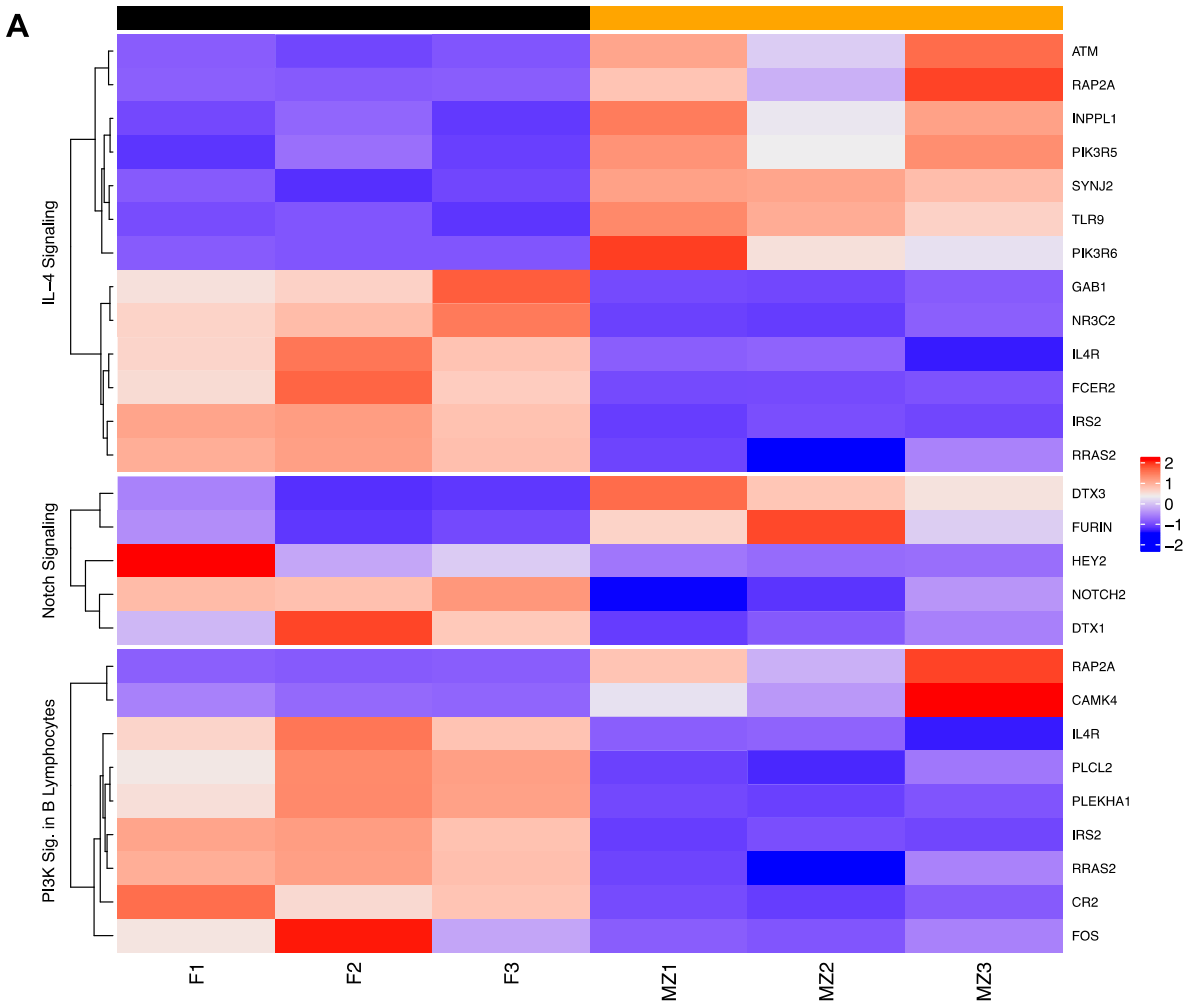


Figure 8. Pathway analysis of tonsil follicular B cells and marginal zone B cells. Heatmap display of tonsil follicular (three left columns) and marginal zone (three right columns) B cell populations from three individuals. Pathways analyzed were IL-4 signaling, Notch signaling, PI3K signaling in B lymphocytes (A); BCR signaling, Cyclins & cell cycle regulation, Th1/Th2 activation (B); IL-6 signaling, NF-kB signaling, PKA signaling (C).

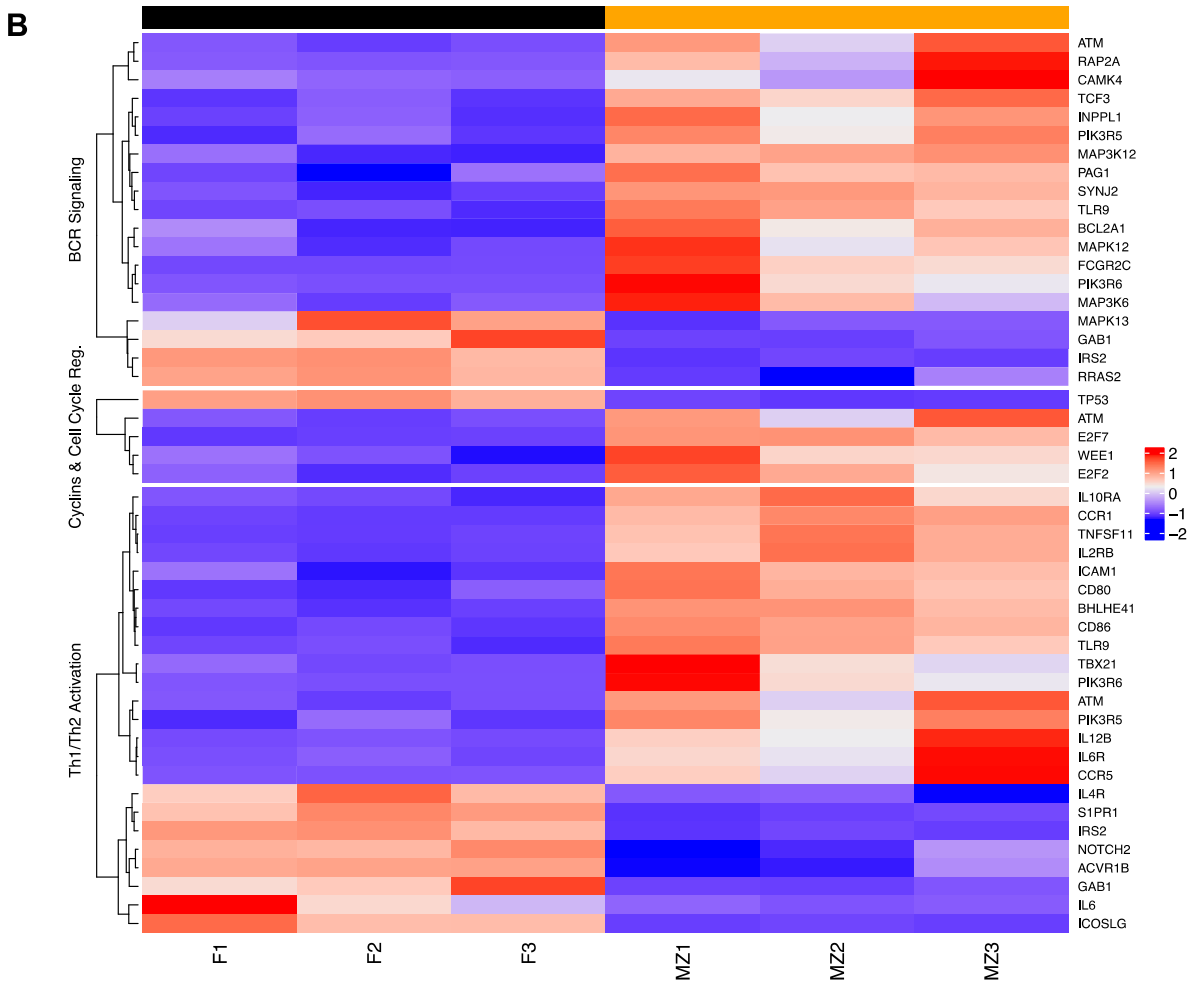


Figure 8. Pathway analysis of tonsil follicular and marginal zone B cells. Heatmap display of tonsil follicular (three left columns) and marginal zone (three right columns) B cell populations from three individuals. Pathways analyzed were IL-4 signaling, Notch signaling, PI3K signaling in B lymphocytes (A); BCR signaling, Cyclins & cell cycle regulation, Th1/Th2 activation (B); IL-6 signaling, NF-kB signaling, PKA signaling (C).

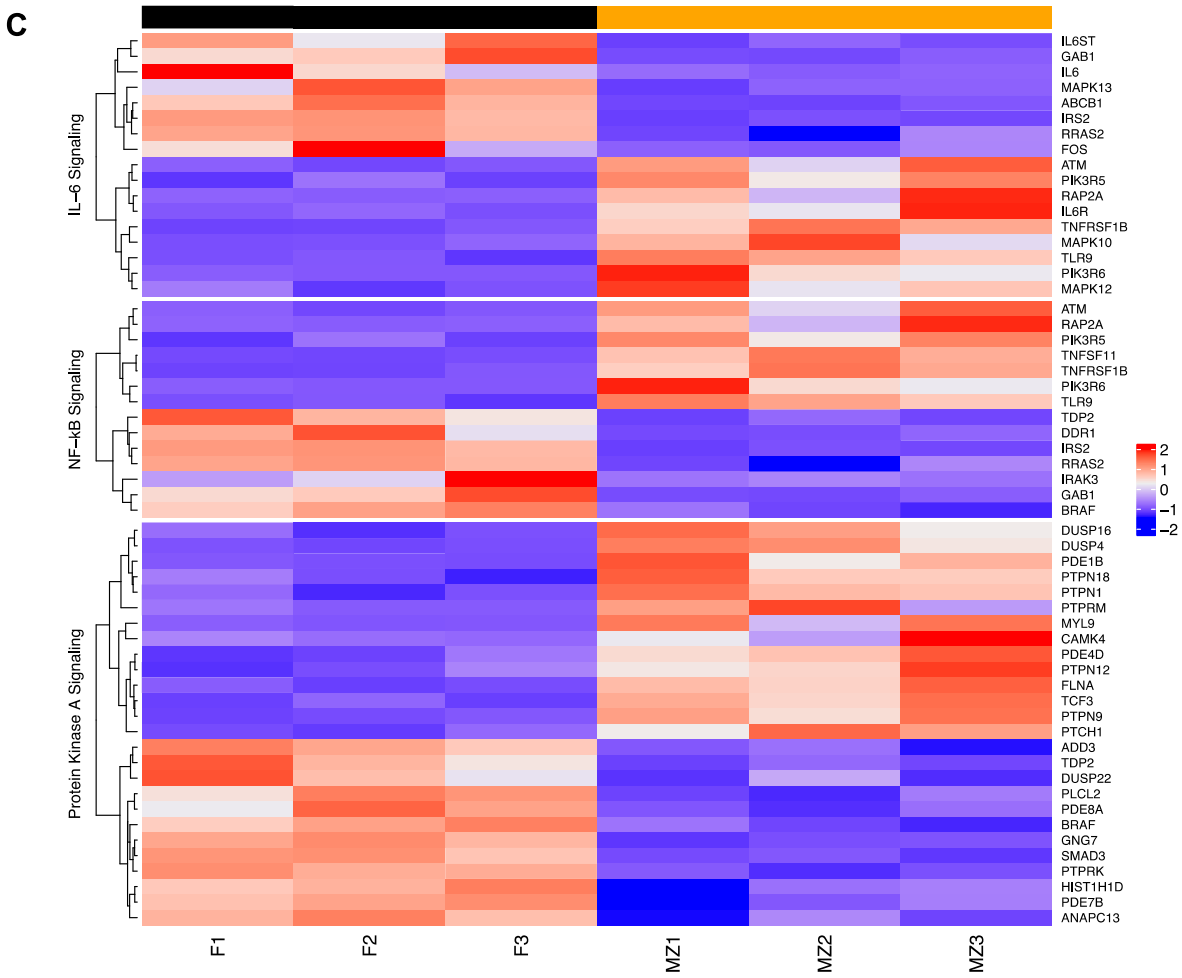


Figure 8. Pathway analysis of tonsil follicular and marginal zone B cells. Heatmap display of tonsil follicular (three left columns) and marginal zone (three right columns) B cell populations from three individuals. Pathways analyzed were IL-4 signaling, Notch signaling, PI3K signaling in B lymphocytes (A); BCR signaling, Cyclins & cell cycle regulation, Th1/Th2 activation (B); IL-6 signaling, NF-kB signaling, PKA signaling (C).

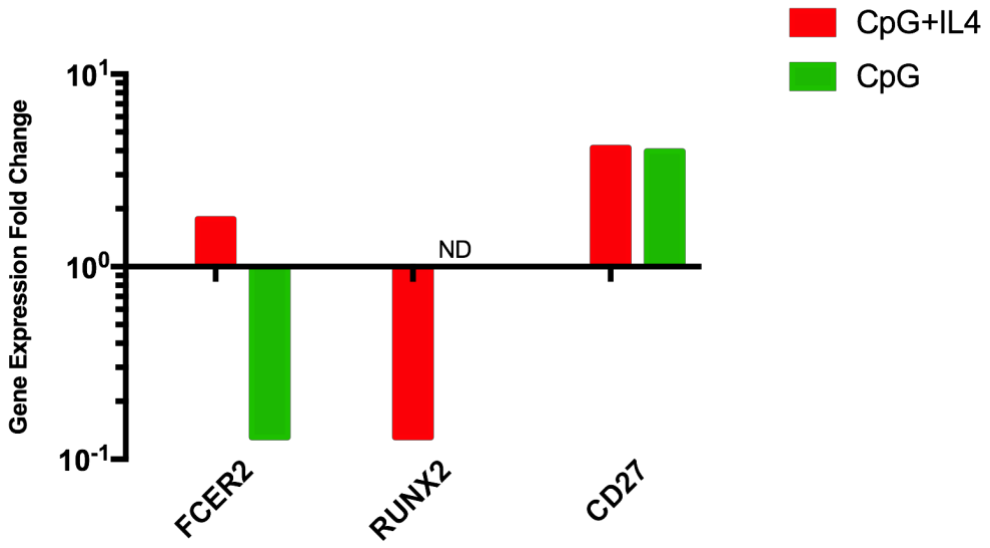


Figure 9. Transitional B cell gene expression following 48h stimulation with CpG + IL-4 or CpG alone. FCER2 (CD23), RUNX2, and CD27 gene expression were analyzed by RT-qPCR.

CHAPTER 5: CONCLUSIONS & FUTURE DIRECTIONS

Vaccines are considered one of the most effective public health interventions for the control of infectious diseases (1). While many highly effective vaccines exist, new and improved vaccines for the prevention of infectious diseases of major public health concern are still necessary (2). The addition of adjuvants has helped to improve the immunogenicity and efficacy of vaccines (3-7). Specifically, PRR ligands are being incorporated as vaccine adjuvants due to their immunostimulatory potential (8-10). Several vaccines in experimental and clinical use utilize PRR ligands alone or in combination with other commonly used vaccine adjuvants (4, 10). Examples of FDA-approved vaccines containing PRR ligands include: SHINGRIX and HEPLISAV-B. SHINGRIX is indicated for the prevention of herpes zoster reactivation in individuals older than 50 years of age (11). SHINGRIX contains varicella zoster glycoprotein E and AS01B, a liposomal adjuvant including MPL (TLR4 ligand) and QS-21 (saponin). HEPLISAV-B is an FDA-approved vaccine for the prevention of hepatitis B in adults 18 years of age and older (12). The HEPLISAV-B vaccine contains CpG 1018 as its adjuvant. HEPLISAV-B is the first FDA-approved vaccine whose adjuvant is composed of a PRR ligand alone. The previously approved hepatitis B vaccine for use in children and adults utilizes alum as its adjuvant.

PRR ligands may activate many immune cell types including dendritic cells, monocytes, and B cells (13-17). While the effects of several PRR ligands have been tested on dendritic cells and monocytes in the mouse and human (18), the direct effect of PRR ligands on human B cells is unclear and understudied. More specifically, there is little to no literature defining the direct role of PRR ligands on human B cell maturation and

activation. In contrast to alum, a commonly utilized vaccine adjuvant, PRR ligands have defined receptors and signaling pathways. As such, the incorporation of PRR ligands as vaccine adjuvants will allow for more specific and defined responses that may involve direct effects on B cell subpopulations.

In humans, transitional B cells are a prominent B cell subset during infancy and early childhood (birth – 4 years of age) (19). Additionally, this developmental period coincides with the time routine vaccinations are being administered. It is reasonable to hypothesize that vaccines containing PRR ligands as adjuvants may affect the mature B cell compartment by driving the maturation of transitional B cells. Select PRR ligand-induced changes in the mature B cell compartment, due to direct effects on the maturation of transitional B cells, may guide rational vaccine adjuvant design in the future.

The overall goal of this work was to determine whether PRR ligands could induce B cell maturation from transitional to mature naïve B cells in humans. Additionally, do different PRR ligands influence the follicular (Fo) or marginal zone (MZ) B cell fate decision? If so, how can we measure these effects? We hypothesized that PRR ligands can drive B cell maturation from the transitional B cell stage and that select PRR ligands are capable of influencing transitional B cells to differentiate into either Fo B cells or MZ B cells.

Human transitional B cells can be identified using several surface markers including CD24 and CD38. Previous literature relied primarily on high CD24 and CD38 expression, however, some late stage transitional B cells express low levels of CD24 and CD38 (20, 21). Recently, the lack of ABCB1 expression has been associated with the transitional B cell subset (22) while mature, naïve B cells are characterized as expressing ABCB1. Rhodamine 123 (R123) is a cell-permeable, fluorescent dye that is excreted via the ABCB1 transporter. Therefore, transitional B cells retain high levels of R123. We utilized the expression of several cell surface markers and R123 fluorescence to isolate transitional B cells from human cord blood. Additionally, high CD23 expression and R123 extrusion were indicative of transitional B cell maturation into naïve B cells.

Our studies confirmed previous work that IL-4 was necessary for transitional B cell survival. After two days of culture, IL-4-supplemented transitional B cell cultures contained a small population of $CD23^{hi}$ follicular-like B cells. The highest frequency of $CD23^{hi}$ Fo-like B cells were produced in transitional B cell cultures stimulated with R848, iE-DAP, and CpG in the presence of IL-4. In contrast, transitional B cells stimulated with TDB plus IL-4 or CpG alone resulted in little to no increase in $CD23^{hi}$ Fo-like B cells. Rather the majority of these cells expressed intermediate levels of CD23 while a minority were $CD23^{-}$ $CD21^{+}$ B cells. These $CD23^{-}$ $CD21^{+}$ B cells bore a phenotypic resemblance to MZ-like B cells (23). Previous literature has shown that CpG (TLR9 ligand) drove human transitional B cells to develop into IgM memory B cells, a $CD27^{+}$ subset similar to the MZ population (24). While our study does not confirm the

transitional B cell development into IgM memory B cells or MZ-like B cells, we observed the emergence of a small $CD23^{-}$ $CD21^{+}$ population which may have contained these cells.

As mentioned above, in addition to CD23 acquisition B cell maturation can also be assessed by ABCB1 expression as measured by R123 extrusion. In a separate set of experiments, we used a combination of surface antigen expression and R123 fluorescence to characterize transitional B cell maturation. This allowed us to reconstruct a pattern of B cell maturation induced by stimulation with IL-4 and IL-4 plus R848 as illustrated below (Figure 1).

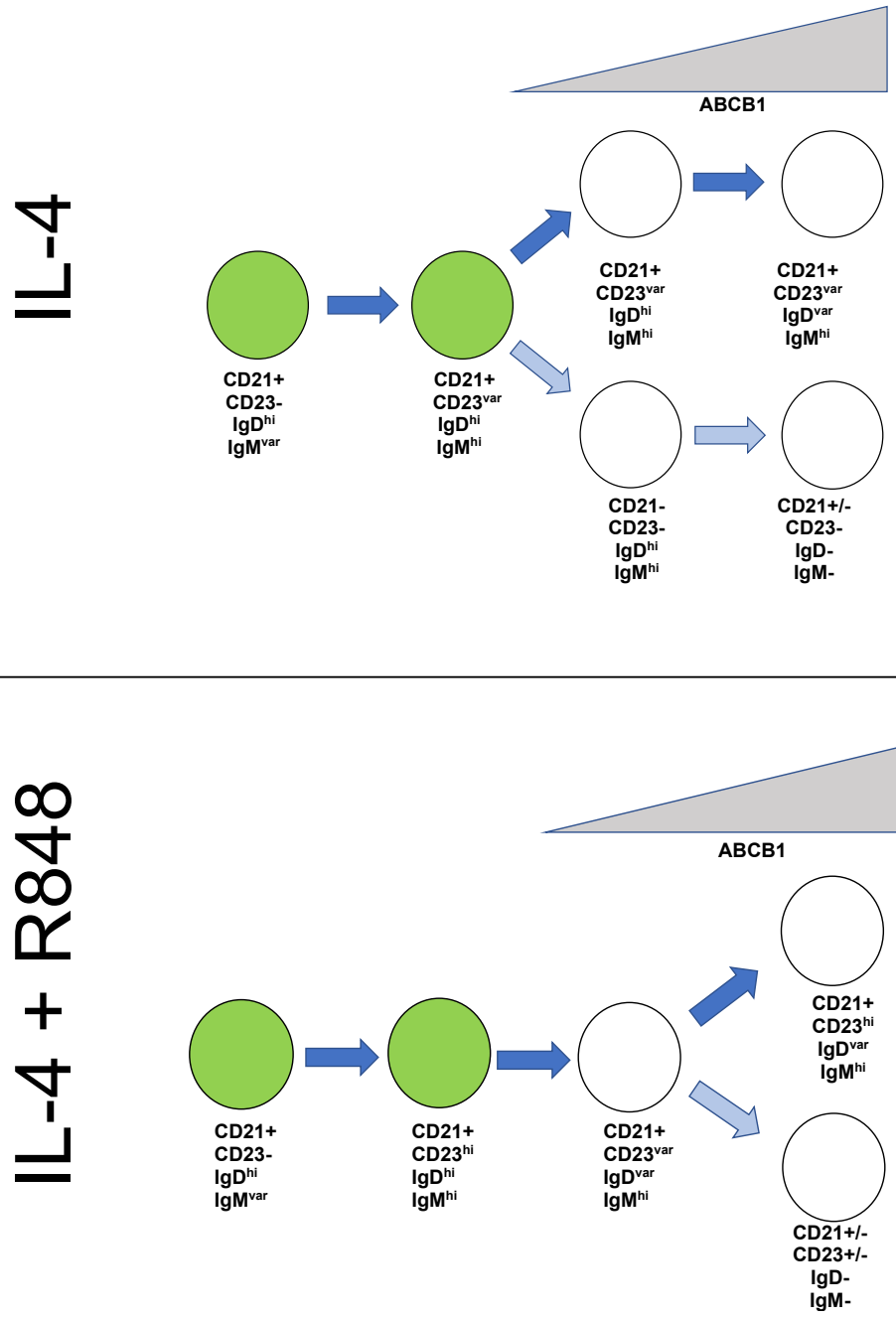


Figure 1. Pattern of transitional B cell maturation induced by either IL-4 or IL-4 + R848. R123 retention is indicated by dark green ($R123^{hi}$), light green ($R123^{int}$), and no green ($R123^{neg}$). Majority populations are indicated by dark blue arrows. Minority populations are indicated by light blue arrows.

Three populations of B cells were observed following IL-4 or IL-4 + R848 stimulation, with R123 high ($R123^{hi}$) cells corresponding to immature, transitional B cells, R123 intermediate to low ($R123^{lo}$) cells corresponding to maturing B cells, and R123 negative ($R123^{neg}$) cells corresponding to fully mature, naïve B cells. In cultures receiving either stimuli, residual $R123^{hi}$ transitional B cells already acquired CD23 on their surface and expressed high levels of IgM, indicating that transitional B cells become CD23 positive and upregulate surface IgM prior to expressing ABCB1. $R123^{neg}$, mature B cells recovered from stimulated cultures were heterogeneous in surface phenotype, consisting of a majority population of $CD23^{hi}$ IgM^{hi} IgD^{var} B cells and minority populations of $CD23^{-}$ IgM^{+} IgD^{+} B cells and $CD23^{-}$ IgM^{-} IgD^{-} B cells. The $CD23^{hi}$ IgM^{hi} IgD^{var} phenotype corresponds to that of naïve, Fo B cells. The $CD23^{-}$ IgM^{+} IgD^{+} population resembles MZ B cells which may be derived directly from transitional B cells or from $CD23^{+}$ precursors in the naïve B cell population. While CD23 expression is generally considered a marker of human follicular B cells, it is also found on human splenic MZ precursors (25). It is interesting to note that previous literature has supported the capacity of Fo B cells to develop into MZ precursors (26). In summary, stimulation of transitional B cells with IL-4 and IL-4 + R848 appears to result in a mixed population of late-stage transitional B cells, Fo B cells, and MZ-like precursors. However, the representation of these populations may be dependent on the nature of the stimuli, as IL-4 was less effective at generating fully-mature B cells than IL-4 and R848 in combination. In our study transitional B cells stimulated with R848/CpG + IL-4 induced a $CD23^{+}$ follicular-like polarizing condition with a minority population of $CD23^{-}$ MZ-like B

cells, while transitional B cells stimulated with CpG alone remain mostly $CD23^-$ and resemble MZ-like B-cells

Previous literature has demonstrated that murine Fo and MZ B cells can be identified by genes related to Notch signaling and several genes associated with B cell maturation in the mouse (BTK, ID2, ID3, ASB2, TCF3) (27-31). Notch signaling plays a key role in the cell fate decision of transitional B cells into mature Fo or MZ B cells (32, 33). While Notch signaling inhibits Fo B cell development, it is a necessary signaling pathway in MZ B cell development. When we measured gene expression of cells from our follicular-like polarizing condition as compared to cells from our marginal zone-like polarizing condition, we saw variable expression of several Notch-related genes. However, NOTCH2NL, a truncated form of NOTCH2 with inhibitory properties (34), is consistently upregulated in both Fo-like and MZ-like polarizing conditions. The elevated expression of NOTCH2NL suggests that transitional B cells stimulated with PRR ligands are differentiating in a NOTCH-independent manner.

In addition to NOTCH-related genes, we evaluated genes known to be associated with murine follicular B cells (BTK, ID3) and marginal zone B cells (ID2, ASB2, TCF3). We confirmed gene expression in purified human tonsil follicular and marginal zone B cells. We observed that transitional B cells under our Fo-like polarizing condition express BTK, similar to human tonsil follicular B cells. We observed that transitional B cells under our MZ-like polarizing condition express ASB2 and TCF3. These gene expression data in combination with surface phenotype and ABCB1 data suggest that

PRR stimulation, in the presence of IL-4, can drive the maturation of translational B cells into fully mature, naïve B cells.

Previous literature has demonstrated the difficulty in defining mature human marginal zone B cells by cell surface phenotype due to the heterogeneity within the population based on tissue location (35-38). Analyses of tonsil MZ-like B cells and B cells that reside in tonsillar MZ-like regions have shown that FcRL4 is expressed on these cells (39-42). A subset of our $CD23^-$ IgM^+ B cells expressed FcRL4 (Chapter 4), similar to previously described tonsillar MZ-like B cells. Therefore, for the remaining studies we decided to utilize the $CD23^-$ IgM^+ $FcRL4^+$ phenotype to define the MZ-like population. Additionally, we evaluated whether IL-4 and PRR ligands have the capacity to induce FcRL4 expression in maturing transitional B cells. While the starting transitional B cell population was uniformly FcRL4 negative, a percentage of these cells acquired FcRL4 expression when cultured with IL-4 + TDB. TDB + IL-4 promoted non-follicular B cell development (Chapter 3) and increased FcRL4 cells (Chapter 4) which together indicate that TDB + IL-4 may promote maturation of transitional B cells to the MZ phenotype. In culture conditions with other PRR ligands, it may be possible to further increase FcRL4 expression by providing additional tissue signals, such as Notch-signaling pathway interactions.

To enable us to reliably identify marginal zone B cells and distinguish them from transitional B cells and follicular B cells in our in vitro cultures, we set out to identify genes uniquely related to $R123^{hi}$ $CD24^+$ $CD38^+$ human transitional B cells, $CD23^+$ IgD^+

human tonsil follicular B cells, and $CD23^-$ IgM^+ $FcRL4^+$ human tonsil marginal zone-like B cells using RNASeq analysis. We found that transitional B cells were more transcriptionally active compared to follicular B cells. Additionally, pathway analysis identified many differentially expressed genes that were uniquely expressed in either the Fo or MZ subset. We analyzed FCER2 gene expression as a positive control since FCER2 encodes for CD23, one of the differentially expressed markers we used to sort Fo and MZ-like subsets. RT-PCR analysis confirmed that $CD23^+$ IgD^+ Fo B cells express ADAM28, BATF, RUNX1 and TNFRSF17, while CD23- IgM+ FcRL4+ tonsil MZ-like B cells express CD27, RUNX2, TBX21. Preliminary analysis evaluating in vitro maturation of transitional B cells stimulated with CpG and CpG + IL-4 indicated an increased expression of the Fo-B cell associated FCER2/CD23 gene in cultures stimulated with CpG + IL-4 and a down-regulation of this gene in cultures stimulated with CpG alone. Furthermore, RUNX2, a probable MZ-specific gene, was down-regulated in cultures stimulated with CpG + IL-4. These results confirm our phenotypic analyses suggested that CpG favored the development of MZ-like B cells while CpG + IL-4 promoted the development of Fo-like B cells. Additional studies to confirm this observation with a larger panel of genes associated with these two B cell subsets are in progress. Importantly, the pattern of gene expression observed in this study supports our conclusion that a subpopulation of transitional B cells can be induced to mature into both Fo-like and MZ-like B cells under specific stimulation conditions.

To the best of our knowledge, this is the first study to look at the effect of several PRR ligands on the maturation of human transitional B cells into mature, naïve B cells and to

examine the follicular versus marginal zone B cell fate decision. The PRR ligands we chose to investigate have been incorporated in experimental and clinical vaccines as adjuvants. Therefore, this study is highly relevant for the rational design of future vaccine adjuvants.

In support of our original hypothesis for Specific Aim 1, we found the TLR7/8, NOD1, Mincle, and TLR9 ligands have the most pronounced effect on transitional B cell maturation. In addition to influencing the development of Fo B cells and MZ B cells, these PRR ligands may also determine whether the antibody response induced will favor the development of long-lived plasma cells or short-lived plasma cells, respectively.

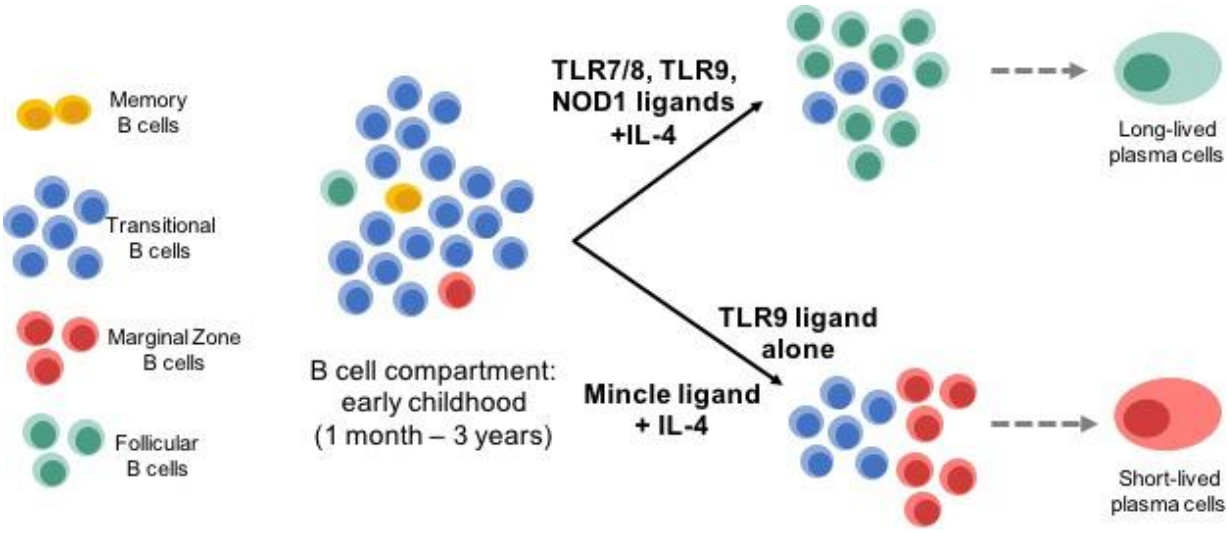


Figure 2. Summary of the effect of select PRR ligands on the early childhood B cell compartment and its influence on the resulting immune response.

Therefore, vaccines with adjuvants that include TLR7/8, NOD1, Mincle, or TLR9 ligands may alter the B cell compartment through their influence on transitional B cell maturation in the very young. The rational design of vaccine adjuvants that take into consideration the effect of PRR ligands on B cell maturation and differentiation may improve the immunogenicity and efficacy of childhood vaccines.

In support of our original hypothesis for Specific Aim 2, we identified several mature Fo and MZ-like B cell-specific genes. These genes may allow for more accurate identification of human Fo and MZ-like subsets, which may be difficult to identify by surface phenotype alone. Furthermore, these differentially expressed genes may allow us to distinguish B cells that are maturing along the Fo or MZ pathway at the gene expression level. The assessment of B cell subset gene expression following vaccination may enhance vaccine evaluation and better predict vaccine immunogenicity and efficacy.

Future Directions

The results of this study have led to several future directions for our research. While this study looked at selected PRR ligands, it would be interesting to examine the effects of the well-known vaccine adjuvant (alum) and newer immunostimulatory molecules (STING ligands and bacterial toxins) on transitional B cell maturation. By assessing the ability of these compounds to drive transitional B cell maturation, we can elucidate the direct effect of various current and potential vaccine adjuvants on human B cells.

This study found that select PRR ligands have the capacity to drive transitional B cell maturation. In addition to driving transitional B cell maturation, PRR ligands have been implicated in the removal of autoreactive B cells from the mature repertoire. Specifically, in the absence of TLR signaling molecules, more autoreactive B cells are found in the mature B cell pool. We hypothesize that other PRR ligands may play a role in removing autoreactive B cells. One approach to test this hypothesis would be to compare the immunoglobulin repertoire in B cells prior to and following stimulation with a panel of PRR ligands.

Our in vitro system allows us to analyze the direct effects of PRR ligands on human transitional B cells. While this is necessary to understand phenotypic and genetic changes in direct response to stimuli, PRR ligand stimulation may have indirect effects on transitional B cell maturation as well. To learn more about these indirect effects, an in vivo system is necessary. Below we propose two approaches for the in vivo analysis of B cell subsets.

Three-dimensional (3D) cell culture systems have become popular in recent years due to their increased relevance to in vivo systems. In a traditional two-dimensional (2D) cell culture system, a monolayer of cells from a cell suspension is seeded on a flat surface like a tissue culture plate or petri dish. This method allows for equal access to nutrients and even culture conditions. However, cells do not exist as 2D monolayers in vivo. As compared to traditional 2D monolayer cell cultures, 3D cell culture systems allow for appropriate cell structure and communication between different cell types. To date, 3D

culture systems have been used to investigate many biological questions including tumor biology, drug resistance/susceptibility, immune response to infection, cell toxicity and hair growth. Previous 3D culture systems have included single cells suspended in agarose or gelatin, organoid structures using scaffolding nanoparticles, and lymphoid tissue sections on gelatin rafts. By isolating human lymphoid tissue for 3D culture, we can examine changes in cell subset frequency in response to PRR stimulation or vaccine formulations containing antigen and adjuvant.

Another approach would be to examine the effect of different vaccine/adjuvant formulations as a result of vaccination. There are currently two licensed vaccines for Hepatitis B. The first formulation to be licensed includes Hepatitis B surface antigen and alum. While more than 90% of the population responds to this formulation, there is a small group of low responders. In response to this low responding population, a second Hepatitis B vaccine formulation was developed containing Hepatitis B surface antigen and CpG (TLR9 ligand). To directly compare the effect of alum and CpG on the transitional B cell population and mature B cell subpopulations, one could compare the frequency of B cell subpopulations in adults who received HepB-alum and adults who received HepB-CpG. This study should be conducted in both a cross-sectional and longitudinal manner. Additionally, since others have noted B cell compartment differences between children and adults. It will be useful to also examine the transitional and mature B cell subpopulations in children before and after routine vaccination with the standard hepatitis B vaccine.

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